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# **Lipoxygenase Activity in Diatoms:**

## **A New Tool to Study the Antiproliferative Effects of Diatoms on Copepod Reproduction**

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Doctor of Philosophy in Life and Biomolecular Sciences

**September 2009**

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## Abstract

The deleterious effects of maternal diatom diets on copepod embryonic development have been puzzling the scientific community for the past 15 years. Since the discovery of the first anti-mitotic compounds, polyunsaturated aldehydes deriving from fatty acid oxidation, our knowledge of the oxylipin metabolism in diatoms has been continuously increasing. Not only have new oxylipin compounds been identified, but it is becoming apparent that oxylipin metabolism is highly complex and specific.

This thesis contributes to the understanding of oxylipin metabolism in diatoms by studying the activity of diatom lipoxygenases, the enzymes responsible for the first oxidative step in transforming polyunsaturated fatty acids into oxylipins. Lipoxygenase activity was measured by a colorimetric and a polarographic assay, and its relationship to oxylipin production was examined. Lipoxygenase metabolism in diatoms was studied under natural conditions during a bloom at sea, under semi-natural conditions during a mesocosm experiment, and in the laboratory, with special emphasis on *Skeletonema marinoi*. The effect of diatom oxylipin production on copepod reproductive success was also examined.

Increased oxylipin production was found with the onset of the stationary phase in culture and during the decline of the mesocosm bloom, which indicates a regulatory role for oxylipins in bloom demise, even though this could not be verified at sea. Lipoxygenase activity and oxylipin production were highly variable and specific to the level of clones. The most important conclusion to be drawn from the present experiments is the lack of correlation between lipoxygenase activity and oxylipin production. This strongly indicates that other compounds still need to be identified and that we do not yet have a complete picture of oxylipin metabolism in diatoms.

# Table of Contents

<b>1</b>	<b>Introduction .....</b>	<b>26</b>
1.1	Plankton in the Marine Environment .....	26
1.2	Diatom-copepod Interactions.....	31
1.2.1	Copepod Reproduction .....	32
1.2.2	Detrimental Impact of Diatom Diets .....	33
1.2.3	Production of Polyunsaturated Aldehydes by Diatoms.....	35
1.2.4	New Insights into Diatom Oxylin Metabolism.....	37
1.3	The Role of Lipoxygenases in Oxylin Production .....	39
1.3.1	General Functions of Lipoxygenases .....	40
1.3.2	Lipoxygenase Activity in Diatoms.....	42
1.4	Aims of the Thesis.....	46
<b>2</b>	<b>Lipoxygenase Metabolism of <i>Skeletonema marinoi</i> during the Diatom Bloom in the Northern Adriatic Sea in 2005 and its Effects on the Reproductive Success of <i>Calanus helgolandicus</i> .....</b>	<b>49</b>
2.1	Introduction.....	50
2.2	Materials and Methods .....	52
2.2.1	General.....	52
2.2.2	Field Phytoplankton Samples .....	53
2.2.2.1	<i>Sample Collection</i> .....	53
2.2.2.2	<i>Sample Preparation</i> .....	54
2.2.3	Colorimetric Lipoxygenase Activity Assay.....	55
2.2.4	Oxylin Analysis.....	58
2.2.4.1	<i>Sample Extraction</i> .....	58
2.2.4.2	<i>Quantification of Aldehydes and Other Oxylin</i> .....	58
2.2.5	Chlorophyll <i>a</i> Determination .....	60
2.2.6	Protein Determination .....	61
2.2.7	Copepod Reproduction.....	61
2.2.8	Copepod Grazing Determined by SEM.....	61

2.3	Results.....	62
2.3.1	Diatom Bloom Development .....	62
2.3.2	Phytoplankton Composition.....	63
2.3.3	Oxylipin Production .....	67
2.3.3.1	<i>Aldehyde Production</i> .....	67
2.3.3.2	<i>Production of Oxylipins Other than Aldehydes</i> .....	69
2.3.3.3	<i>Oxylipin Production</i> .....	71
2.3.4	Lipoxygenase Activity.....	73
2.3.5	Copepod Reproduction.....	75
2.3.5.1	<i>Egg Production Rates</i> .....	75
2.3.5.2	<i>Egg Hatching Success</i> .....	76
2.3.6	Comparison between Years .....	77
2.3.7	Copepod Grazing.....	79
2.4	Discussion .....	84
2.5	Conclusions.....	88
<b>3</b>	<b>Method development.....</b>	<b>89</b>
3.1	Introduction.....	90
3.2	Materials and Methods .....	92
3.2.1	Phytoplankton Culture Samples.....	92
3.2.2	Polarographic Assay.....	93
3.2.3	Colorimetric Assay.....	94
3.2.4	Oxylipin Analysis.....	95
3.2.5	Calibration .....	95
3.3	Results.....	95
3.3.1	Specificity of Lipoxygenase Activity Assays.....	95
3.3.1.1	<i>Polarographic Assay</i> .....	95
3.3.1.2	<i>Colorimetric Assay</i> .....	96
3.3.2	Linearity.....	97
3.3.2.1	<i>Colorimetric Assay</i> .....	97
3.3.2.2	<i>Polarographic Assay</i> .....	100
3.3.3	Stopping the Colorimetric Assay .....	101

3.3.4	pH-variability .....	105
3.3.4.1	<i>Colorimetric Assay</i> .....	105
3.3.4.2	<i>Polarographic Assay</i> .....	105
3.3.4.3	<i>Oxylipin Production</i> .....	106
3.3.5	Comparison of Fresh and Frozen Phytoplankton Samples .....	107
3.3.5.1	<i>Colorimetric Assay</i> .....	107
3.3.5.2	<i>Polarographic Assay</i> .....	109
3.3.5.3	<i>Oxylipin Production</i> .....	110
3.4	Discussion .....	112
3.5	Conclusions .....	117
<b>4</b>	<b>Diversity of Lipoxygenase Metabolism among Different Diatom Species and among Different Clones of the Same Species .....</b>	<b>118</b>
4.1	Introduction .....	119
4.2	Materials and Methods .....	120
4.3	Results .....	121
4.3.1	Species Comparison .....	121
4.3.1.1	<i>Polarographic Assay</i> .....	122
4.3.1.2	<i>Colorimetric Assay</i> .....	125
4.3.1.3	<i>Oxylipin Production</i> .....	128
4.3.2	Comparison of Different Clones of <i>Skeletonema marinoi</i> .....	131
4.3.2.1	<i>Colorimetric Assay</i> .....	132
4.3.2.2	<i>Polarographic Assay</i> .....	133
4.3.2.3	<i>Oxylipin Production</i> .....	134
4.3.2.4	<i>Oxylipin Composition</i> .....	136
4.3.2.5	<i>Other Parameters</i> .....	138
4.4	Discussion .....	140
4.5	Conclusions .....	143

<b>5 Effect of Phosphorus-limitation on Lipoxygenase Activity of <i>Skeletonema marinoi</i> and its Effects on the Reproductive Success of <i>Calanus helgolandicus</i> .....</b>	<b>144</b>
5.1 Introduction.....	145
5.2 Materials and Methods .....	146
5.2.1 Experimental Set-up.....	146
5.2.2 Copepod Reproduction Experiments.....	149
5.2.3 Analysis of Lipoxygenase Activity and Oxylin Production .....	150
5.2.4 Analysis of Particulate Carbon, Nitrogen, and Phosphorus .....	151
5.3 Results.....	152
5.3.1 Nutrient Status.....	152
5.3.2 Oxylin Production .....	153
5.3.2.1 Aldehyde Production .....	153
5.3.2.2 Production of Oxylin Other than Aldehydes .....	154
5.3.2.3 Oxylin Production .....	155
5.3.3 Lipoxygenase Activity.....	156
5.3.4 Copepod Reproduction .....	157
5.4 Discussion .....	163
5.5 Conclusions.....	166
<b>6 Growth-phase Modulated Lipoxygenase Activity in <i>Pseudo-nitzschia delicatissima</i>.....</b>	<b>167</b>
6.1 Introduction.....	168
6.2 Materials and Methods .....	170
6.2.1 Sample Collection .....	170
6.2.2 Sample Analysis.....	171
6.3 Results.....	172
6.3.1 Growth Curve.....	172
6.3.2 Oxylin Production .....	172
6.3.3 Lipoxygenase Activity.....	175
6.3.3.1 Polarographic Assay .....	175

6.3.3.2	<i>Colorimetric Assay</i> .....	176
6.3.4	Oxylipin Composition .....	179
6.4	Discussion .....	181
6.5	Conclusions.....	185
<b>7</b>	<b>Modulated Lipoxygenase Activity during a Bloom of <i>Skeletonema marinoi</i> in a Mesocosm Experiment .....</b>	<b>186</b>
7.1	Introduction.....	187
7.2	Materials and Methods .....	189
7.2.1	Experimental Set-up .....	189
7.2.2	Sample Analysis.....	190
7.2.2.1	<i>Sample Collection</i> .....	191
7.2.2.2	<i>Method Development</i> .....	192
7.2.3	Culture Samples .....	197
7.2.4	Copepod Reproduction.....	197
7.2.5	Data Analysis .....	198
7.3	Results.....	199
7.3.1	Mesocosm Samples .....	199
7.3.1.1	<i>Bloom Development</i> .....	199
7.3.1.2	<i>Lipoxygenase Activity</i> .....	200
7.3.1.3	<i>Oxylipin Production</i> .....	201
7.3.2	Nutrients .....	204
7.3.3	Effects of Oxylipins on Copepods .....	206
7.3.4	Culture Samples .....	212
7.3.4.1	<i>Colorimetric Assay</i> .....	212
7.3.4.2	<i>Polarographic Assay</i> .....	213
7.3.4.3	<i>Oxylipin Production</i> .....	214
7.4	Discussion .....	216
7.5	Conclusions.....	222
<b>8</b>	<b>Conclusions .....</b>	<b>223</b>

8.1 Methodological Aspects .....223

8.2 Uncorrelated Lipoxygenase Activity and Oxylin Production.....225

8.3 Diversity of Oxylin Metabolism among Clones.....227

8.4 Functions of Oxylin in Marine Ecosystems.....229

8.5 Closing Remarks .....231

**9 Bibliography .....232**

**10 Appendix**

Table of tables

Table 2-1: Latitude and longitude of six stations sampled in the Northern Adriatic Sea in 2005. .... 54

Table 2-2: Data points for the colorimetric assay. Blanks (4 mg blank and 8 mg blank) were read immediately, whereas all other samples were read after 20 min. Before reading the samples, 0.5 ml SDS 1% were added, which were already contained in the reagent mix for blanks. .... 57

Table 2-3: Molecular ion mass [m/z] detected in LC-MS analysis of extracts and used for quantification of the hydroxy-acids and epoxyalcohols derived from hexadecatetraenoic acid (HTA), hexadecatrienoic acid (HTrA), and eicosapentaenoic acid (EPA). Abbreviations in brackets indicate oxylipin species identified on the chromatograms in the Appendix. .... 59

Table 2-4 Molecular ion mass [m/z] and retention times [min] detected in GC-MS analysis of extracts and used for quantification of the aldehydes octatrienal, octadienal, heptadienal, and decatrienal, derived from the respective fatty acids hexadecatetraenoic acid (HTA), hexadecatrienoic acid (HTrA), and eicosapentaenoic acid (EPA). Abbreviations in brackets indicate aldehyde species identified on the chromatograms in the Appendix. .... 60

Table 3-1: Centrifugation parameters for collection of culture pellet samples of three diatom species *Chaetoceros affinis*, *Skeletonema marinoi*, and *Thalassiosira rotula*, used in this and the following chapter. .... 93

Table 3-2: Production of aldehydes and oxylipins other than aldehydes (n=1) normalized for cells [fg cell<sup>-1</sup>] in cell lysate of *Skeletonema marinoi* suspended in buffer at pH=6.0 and pH=8.15. Analysis of aldehyde production was carried out on a different sample of *S. marinoi* cell lysate than analysis of production of oxylipins other than aldehydes. .... 107

Table 4-1: Cellular carbon content [pg C cell<sup>-1</sup>] of *Skeletonema marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*, used for calculation of oxylipin production per milligram carbon. Values were obtained from the literature: a) (Carotenuto *et al.* 2002), b) (Koski *et al.* 2008), c) (Ianora and Poulet 1993)..... 121



Table 4-2: Oxylipins, including the aldehydes heptadienal, octadienal, octatrienal, and decatrienal and the epoxyalcohols (EPOX) and hydroxy-acids (OH) produced from hexadecatrienoic acid (C<sub>16:3</sub>) and hexadecatetraenoic acid (C<sub>16:4</sub>), as well as eicosapentaenoic acid (C<sub>20</sub>), produced by *Skeletonema marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*..... 129

Table 5-1: Sampling of the chemostat and the outflow bottle of P-limited and P-replete *Skeletonema marinoi* culture during the course of the replicate experiments 1-3. Highlighted samples produced detectable amounts of aldehydes (see 5.3.2.1)..... 151

Table 5-2: Mean values (±SEM; P-replete: n=6, P-limited: n=4) for particulate organic carbon (POC), nitrogen (PON), and phosphorus (POP) content normalized for cells [pg cell<sup>-1</sup>] and C:N- and N:P-ratios in *Skeletonema marinoi* grown under P-replete and P-limited conditions..... 152

Table 5-3: Parameters (Number of days, initial number of females and survival rate of females [%]) of the incubation experiments carried out with females of *Calanus helgolandicus* feeding on P-limited and P-replete cultures of *Skeletonema marinoi*. ..... 158

Table 7-1: Mesocosm bag treatments (Bags A-F) (cell counts in D-F refer to the initial concentration of *Skeletonema marinoi* upon inoculation) and the parameters analyzed in the various treatments (oxylipin production and reproductive success)..... 190

Table 7-2: Sampling days (days after inoculation) for analysis of oxylipin production in mesocosm bags C, E, and F. .... 191

Table 7-3: Absorbance at 598 nm of *Skeletonema marinoi* cell lysate corresponding to varying amounts of pellet wet weight [mg] in the colorimetric assay..... 193

Table 7-4: Oxygen consumption after 5 min [μmol] in the polarographic assay at pH=8.15 in *Skeletonema marinoi* cell lysate corresponding to varying amounts of pellet wet weight [mg] incubated without (lysate) and with EPA (lysate+EPA)..... 194

Table 7-5: Absorbance at 655 nm of *Skeletonema marinoi* cell lysate corresponding to varying amounts of pellet wet weight [mg] in the assay for protein determination..... 194

Table 7-6: Chlorophyll *a* [ng] determined in *Skeletonema marinoi* cell lysate, depending on pellet wet weight used in the assay [mg]. ..... 195

Table 7-7: Comparison of Conway culture medium used in Bergen and f/2 medium used at SZN in respect to macro- (NO<sub>3</sub>, PO<sub>4</sub>) and micro- (Fe, Mn, Zn, Co, Mo, Cu) nutrient final concentrations [μM]. ..... 216

# Table of figures

Figure 1-1: Diatoms from Ernst Haeckel’s “Kunstformen der Natur” (1904). ..... 28

Figure 1-2: Copepods from Ernst Haeckel’s “Kunstformen der Natur” (1904). ..... 30

Figure 1-3: The classical marine food web; the box highlights the interactions between phytoplankton and copepods (Hardy 1959). ..... 31

Figure 1-4: Production of the polyunsaturated aldehydes (PUAs) octatrienal and octadienal from C<sub>16</sub>-fatty acids released from glycolipids, and production of heptadienal and decatrienal from C<sub>20</sub>-fatty acids released from phospholipids; adapted from d’Ippolito *et al.* (2004) and Pohnert (2005). ..... 36

Figure 1-5: 9-lipoxygenase (LOX) activity on C<sub>16</sub>-fatty acids (HTA, HTrA) in diatoms; adapted from Barofsky and Pohnert (2007) and Andreou *et al.* (2009) (AOS: allene oxide synthase, HPL: hydroperoxide lyase). ..... 44

Figure 1-6: Selective overview of oxylipin metabolism of the C<sub>20</sub>-fatty acid eicosapentaenoic acid (EPA) in diatoms; adapted from Andreou *et al.* (2009) (LOX: lipoxygenase, AOS: allene oxide synthase, HPL: hydroperoxide lyase, HPEPA: hydroperoxy-eicosapentaenoic acid). ..... 45

Figure 2-1: Oxidative coupling due to fatty acid hydroperoxides (FAHs) of 3-methyl-2-benzothiazolinone (MBTH) and 3-(dimethylamino)-benzoic acid (DMAB) catalyzed by haemoglobin. .... 51

Figure 2-2: Mean diatom cell concentrations (±SEM, n=6) of stations 1-6 [cells ml<sup>-1</sup>] in the phytoplankton net samples in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19). ..... 63

Figure 2-3: Phytoplankton composition at stations 1-6 in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19). Species identified included *Skeletonema marinoi*, *Chaetoceros spp.*, *Pseudo-nitzschia spp.*, *Thalassiosira rotula*, and other minor phytoplankton species including non-diatoms. .... 65

Figure 2-4: Aldehyde production (n=1) normalized for diatom cells [pg cell <sup>-1</sup> ] in phytoplankton samples collected at stations 1-6 in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).....	68
Figure 2-5: Aldehyde production (n=1) normalized for protein [μg (mg prot) <sup>-1</sup> ] in phytoplankton samples collected at stations 1-6 in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).....	68
Figure 2-6: Production of oxylipins other than aldehydes (n=1) normalized for diatom cells [pg cell <sup>-1</sup> ] in phytoplankton samples collected at stations 1-6 in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19). ....	69
Figure 2-7: Mean production (±SEM, n=6) of oxylipins other than aldehydes of stations 1-6 normalized for protein [μg (mg prot) <sup>-1</sup> ] in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).....	70
Figure 2-8: Mean oxylipin production (±SEM, n=6) of stations 1-6 normalized for diatom cells [pg cell <sup>-1</sup> ] in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).....	71
Figure 2-9: Mean oxylipin production (±SEM, n=6) of stations 1-6 normalized for protein [μg (mg prot) <sup>-1</sup> ] in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19). ....	72
Figure 2-10: Mean fatty acid hydroxide (FAH) production (±SEM, n=6) of stations 1-6 normalized for protein [μmol (mg prot) <sup>-1</sup> ] determined with the colorimetric assay in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19). FAH production was calculated from FAH concentrations in cell lysates after 20 min without considering blanks. ....	73
Figure 2-11: Mean fatty acid hydroxide (FAH) production (±SEM, n=6) of stations 1-6 normalized for chlorophyll <i>a</i> [μmol (ng chl <i>a</i> ) <sup>-1</sup> ] determined with the colorimetric assay in February-May 2005 (numbers on x-axis indicate weeks of the year; sample analysis was	

only carried out up to week 11). FAH production was calculated from FAH concentrations in cell lysates after 20 min without considering blanks..... 74

Figure 2-12: Mean egg production rates (EPR) ( $\pm$ SEM, n=6) of *Calanus helgolandicus* females [eggs (fem d)<sup>-1</sup>] collected at stations 1-6 in February-April 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15). ..... 75

Figure 2-13: Mean hatching success ( $\pm$ SEM, n=6) of copepod eggs produced by *Calanus helgolandicus* females [%] collected at stations 1-6 in February-April 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15)..... 76

Figure 2-14: Yearly mean oxylipin production ( $\pm$ SEM, n=6) normalized for diatom cells [pg cell<sup>-1</sup>] in phytoplankton samples collected at stations 1-6 during the late winter diatom blooms in 2003, 2004, and 2005. .... 77

Figure 2-15: Yearly mean hatching success ( $\pm$ SEM, n=6) of copepod eggs produced by *C. helgolandicus* females [%] collected at stations 1-6 during the late winter diatom blooms in 2003, 2004, and 2005. .... 78

Figure 2-16: Yearly mean oxylipin production ( $\pm$ SEM, n=6) normalized for protein [ $\mu$ g (mg prot)<sup>-1</sup>] in phytoplankton samples collected at stations 1-6 during the late winter diatom blooms in 2003, 2004, and 2005..... 79

Figure 2-17: SEM photograph of a faecal pellet produced by a *Calanus helgolandicus* female collected at the beginning of the diatom bloom in 2009 (March 4<sup>th</sup>, station 2). The red circle shows an intact cell of *Skeletonema marinoi*..... 80

Figure 2-18: SEM photograph of a faecal pellet produced by a *Calanus helgolandicus* female collected on March 16<sup>th</sup> 2009 from station 2..... 81

Figure 2-19: SEM photograph of a faecal pellet produced by a *Calanus helgolandicus* female collected on March 24<sup>th</sup> 2009 from station 2..... 82

Figure 2-20: SEM photograph of a faecal pellet produced by a *Calanus helgolandicus* female collected on March 30<sup>th</sup> 2009 from station 2..... 83

Figure 3-1: Oxidation of polyunsaturated fatty acids (PUFAs) to fatty acid hydroperoxides (FAHs) by lipoxygenase (LOX) via insertion of a dioxygen molecule (O<sub>2</sub>)..... 91

Figure 3-2: Mean oxygen consumption rate (+SEM, n=2 except *Skeletonema marinoi*: n=12) [μmol O<sub>2</sub> (mg prot min)<sup>-1</sup>] in cell lysate (lysate) and in cell lysate incubated with EPA (0.2 mM) (lysate+EPA) of *Thalassiosira weissflogii*, *Prorocentrum minimum*, and *S. marinoi*, including a boiled sample of *S. marinoi* measured with the polarographic assay at pH=8.15..... 96

Figure 3-3: Mean fatty acid hydroperoxide (FAH) production (+SEM, n=2) [μM] from varying amounts of pure lipoxygenase (LOX) enzyme [unit] incubated with 0.4 mM eicosapentaenoic acid only (+EPA) and with EPA and the LOX inhibitor (0.05 mM) nordihydroguaiaretic acid (+EPA +NDGA) determined with the colorimetric assay. .... 97

Figure 3-4: Relationship between mean absorbance (±SEM, n=2) at 598 nm and concentration of eicosapentaenoic acid hydroperoxide (FAH) [μM] in the colorimetric assay..... 98

Figure 3-5: Relationship between mean fatty acid hydroperoxide (FAH) concentration (±SEM, n=1-3 depending on LOX amount) [μM] and amount of lipoxygenase (LOX) [unit] incubated with EPA (0.4 mM) in the colorimetric assay. .... 99

Figure 3-6: Relationship between mean fatty acid hydroperoxide (FAH) concentration (±SEM, n=1-2 depending on pellet wet weight) [μM] and *Skeletonema marinoi* pellet wet weight [mg] in the colorimetric assay. .... 100

Figure 3-7: Relationship between mean oxygen consumption rate (±SEM, n=1-3 depending on LOX amount) [nmol O<sub>2</sub> min<sup>-1</sup>] and amount of lipoxygenase (LOX) [unit] incubated with EPA (0.4 mM) in the polarographic assay at pH=8.15..... 101

Figure 3-8: Mean absorbance (+SEM, n=2) at 598 nm of a boiled cell lysate and a normally lysed cell suspension of *Skeletonema marinoi* at 0, 5, and 20 min after the start of the colorimetric assay. .... 102

Figure 3-9: Mean absorbance (+SEM, n=2) at 598 nm of samples read 0, 20, and 40 min after stopping the colorimetric assay by boiling samples. The colorimetric assay was carried out with cell lysate of *Skeletonema marinoi*..... 103

Figure 3-10: Mean absorbance (+SEM, n=2) at 598 nm of samples read 0, 20, 40, and 50 min after stopping the colorimetric assay by addition of increasing concentrations of SDS (0.3, 2, and 7%). The colorimetric assay was carried out with a cell lysate of *Skeletonema marinoi*. ..... 104

Figure 3-11: Mean absorbance (+SEM, n=2) at 598 nm of a cell lysate of *Skeletonema marinoi* measured in the colorimetric assay carried out at various pH (pH=6.0, 8.15, and 9.0). Absorbance values were not corrected for blank values. .... 105

Figure 3-12: Mean oxygen consumption rate (+SEM; pH=6.0: n=2, pH=8.15: n=4, pH=9.0: n=1) [ $\mu\text{mol O}_2 \text{ (mg prot min)}^{-1}$ ] of *Skeletonema marinoi* cell lysate upon EPA-addition (0.2 mM) determined polarographically at various pH (pH=6.0, 8.15, and 9.0)..... 106

Figure 3-13: Mean production (+SEM, n=4) of fatty acid hydroperoxide (FAH) normalized for protein [ $\mu\text{mol (mg prot)}^{-1}$ ] in fresh and frozen samples of *Chaetoceros affinis*. Detection of FAH was carried out with the colorimetric assay. .... 108

Figure 3-14: Mean production (+SEM, n=4) of fatty acid hydroperoxide (FAH) normalized for protein [ $\mu\text{mol (mg prot)}^{-1}$ ] in fresh and frozen samples of *Skeletonema marinoi*. Detection of FAH was carried out with the colorimetric assay. .... 108

Figure 3-15: Mean oxygen consumption rate (+SEM, n=4) [ $\mu\text{mol O}_2 \text{ (mg prot min)}^{-1}$ ] upon EPA-addition determined polarographically in fresh and frozen samples of *Chaetoceros affinis* at pH=8.15..... 109

Figure 3-16: Mean oxygen consumption rate (+SEM, n=4) [ $\mu\text{mol O}_2 \text{ (mg prot min)}^{-1}$ ] upon EPA-addition determined polarographically in fresh and frozen samples of *Skeletonema marinoi* at pH=8.15..... 110

Figure 3-17: Mean oxylipin production (+SEM, n=4) normalized for cells [ $\text{pg cell}^{-1}$ ] in fresh and frozen samples of *Chaetoceros affinis*. .... 111

Figure 3-18: Mean oxylipin production (+SEM, n=4) normalized for cells [ $\text{fg cell}^{-1}$ ] in fresh and frozen samples of *Skeletonema marinoi*. .... 111

Figure 4-1: Mean oxygen consumption rate [ $\mu\text{mol O}_2 \text{ (mg prot min)}^{-1}$ ] upon EPA-addition (0.2 mM) (+SEM, n=9) in *Skeletonema marinoi* determined with the polarographic assay at two pH-values (pH=6.0 and pH=8.15). ..... 122

Figure 4-2: Mean oxygen consumption rate [ $\mu\text{mol O}_2 \text{ (mg prot min)}^{-1}$ ] upon EPA-addition (0.2 mM) (+SEM, n=3) in *Thalassiosira rotula* determined with the polarographic assay at two pH-values (pH=6.0 and pH=8.15). ..... 123

Figure 4-3: Mean oxygen consumption rate [ $\mu\text{mol O}_2 \text{ (mg prot min)}^{-1}$ ] upon EPA-addition (0.2 mM) (+SEM, n=3) in *Chaetoceros affinis* determined with the polarographic assay at two pH-values (pH=6.0 and pH=8.15). ..... 124

Figure 4-4: Mean oxygen consumption rate [ $\mu\text{mol O}_2 \text{ (mg prot min)}^{-1}$ ] upon EPA-addition (+SEM; *Skeletonema marinoi*: n=12, *Thalassiosira rotula*: n=5, *Chaetoceros affinis*: n=3) determined with the polarographic assay at pH=8.15 in *S. marinoi*, *T. rotula*, and *C. affinis*. ..... 125

Figure 4-5: Mean concentrations (+SEM, n=3 except *Skeletonema marinoi* n=15) of fatty acid hydroperoxide (FAH) [ $\mu\text{mol (mg prot)}^{-1}$ ] determined with the colorimetric assay in blanks (0 min), in cell lysate of *S. marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis* (20 min) and in cell lysate upon EPA-addition (+EPA). ..... 126

Figure 4-6: Mean oxygen consumption rate [ $\text{nmol O}_2 \text{ (mg prot min)}^{-1}$ ] upon EPA-addition (+SEM; n=3, except *Skeletonema marinoi*: n=8) determined with the polarographic assay at pH=6.0 in *S. marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*. ..... 128

Figure 4-7: Mean production (+SEM; n=3 except *Skeletonema marinoi*: n=12) of aldehydes and other oxylipins normalized for cells [ $\text{fg cell}^{-1}$ ] in *S. marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*. ..... 130

Figure 4-8: Mean production (+SEM; n=3 except *Skeletonema marinoi*: n=12) of aldehydes and other oxylipins normalized for cell carbon [ $\mu\text{g (mg C)}^{-1}$ ] in *S. marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*. ..... 131

Figure 4-9: Mean concentrations (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) of fatty acid hydroperoxide (FAH) [ $\mu\text{mol (mg prot)}^{-1}$ ] determined with the colorimetric assay



in blanks (0 min), in cell lysate (20 min) and in cell lysate upon EPA-addition (+EPA) of the <i>Skeletonema marinoi</i> clones isolated in 1997, 2003, 2004, and 2005.....	132
Figure 4-10: Mean oxygen consumption rate [nmol O <sub>2</sub> (mg prot min) <sup>-1</sup> ] upon EPA-addition (+SEM; 1997: n=4, 2003: n=5, 2004: n=3, 2005, n=2) determined with the polarographic assay at pH=6.0 in the <i>Skeletonema marinoi</i> clones isolated in 1997, 2003, 2004, and 2005. ....	133
Figure 4-11: Mean oxygen consumption rate [μmol O <sub>2</sub> (mg prot min) <sup>-1</sup> ] upon EPA-addition (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) determined with the polarographic assay at pH=8.15 in the <i>Skeletonema marinoi</i> clones isolated in 1997, 2003, 2004, and 2005. ....	134
Figure 4-12: Mean aldehyde production (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) normalized for cells [fg cell <sup>-1</sup> ] in the <i>Skeletonema marinoi</i> clones isolated in 1997, 2003, 2004, and 2005.....	135
Figure 4-13: Mean production (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) of oxylipins other than aldehydes normalized for cells [fg cell <sup>-1</sup> ] in the <i>Skeletonema marinoi</i> clones isolated in 1997, 2003, 2004, and 2005. ....	135
Figure 4-14: Mean oxylipin production (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) normalized for cells [fg cell <sup>-1</sup> ] in the <i>Skeletonema marinoi</i> clones isolated in 1997, 2003, 2004, and 2005.....	136
Figure 4-15: Mean oxylipin composition (+SEM; 1997; n=6, 2003: n=9, 2004 and 2005: n=3) normalized for cells [fmol cell <sup>-1</sup> ] in the <i>Skeletonema marinoi</i> clones isolated in 1997, 2003, 2004, and 2005 (EPOX and OH: epoxyalcohols and hydroxy-acids derived from C <sub>16:3</sub> -, C <sub>16:4</sub> -, and C <sub>20</sub> -fatty acids, respectively).....	137
Figure 4-16: Mean contributions (+SEM; 1997; n=6, 2003: n=9, 2004 and 2005: n=3) of C <sub>16</sub> - and C <sub>20</sub> -derivatives to oxylipin composition [fmol cell <sup>-1</sup> ] in the <i>Skeletonema marinoi</i> clones isolated in 1997, 2003, 2004, and 2005. ....	138
Figure 4-17: Mean protein concentration (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) [mg prot ml <sup>-1</sup> ] in cell lysate of the <i>Skeletonema marinoi</i> clones isolated in 1997, 2003, 2004, and 2005. ....	139

Figure 4-18: Mean growth rate (+SEM, 1997: n=3, 2003: n=3, 2004: n=6, 2005: n=4) [ $d^{-1}$ ] of the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005..... 139

Figure 5-1: On the left, the entire chemostat set-up in the culture chamber with the culture medium bottle on the top, the chemostat bottle on the middle, and the outflow bottle on the bottom shelf. On the right, a close-up of the chemostat bottle with inflowing medium, effluent culture and bubbling. Photos are courtesy of François Ribalet..... 147

Figure 5-2: Mean maximum growth rate ( $\mu_{max}$ ) ( $\pm$ SEM, n=3 except day 2: n=2) of *Skeletonema marinoi* in the chemostat. .... 149

Figure 5-3: Mean aldehyde production (+SEM; P-limited: n=5, P-replete: n=7) normalized for cells [ $fg\ cell^{-1}$ ] in P-limited and P-replete cultures of *Skeletonema marinoi*. Data for samples of chemostat and outflow bottles were pooled. .... 153

Figure 5-4: Mean production of oxylipins other than aldehydes (+SEM; P-limited chemostat: n=3, P-limited outflow: n=2, P-replete chemostat: n=3, P-replete outflow: n=4) normalized for cells [ $fg\ cell^{-1}$ ] in chemostat and outflow bottles of P-limited and P-replete cultures of *Skeletonema marinoi*. .... 154

Figure 5-5: Mean oxylipin production (+SEM, P-limited chemostat: n=3, P-limited outflow: n=2, P-replete chemostat: n=3, P-replete outflow: n=4) normalized for cells [ $fg\ cell^{-1}$ ] in chemostat and outflow bottles of P-limited and P-replete cultures of *Skeletonema marinoi*. .... 155

Figure 5-6: Fatty acid hydroperoxide (FAH) production [ $\mu mol\ (mg\ prot)^{-1}$ ] (n=1) determined with the colorimetric assay in chemostat and outflow bottles of P-limited and P-replete cultures of *Skeletonema marinoi*. FAH production was defined as the difference in FAH concentrations between cell lysates after 20 min and blanks. .... 156

Figure 5-7: Oxygen consumption rate [ $\mu mol\ O_2\ (mg\ prot\ min)^{-1}$ ] upon EPA-addition (0.4 mM) to cell lysate (n=1) determined with the polarographic assay at pH=8.15 in the chemostat bottle of P-replete culture and the outflow bottle of P-limited culture of *Skeletonema marinoi*. .... 157

Figure 5-8: Mean egg production rates (EPR) ( $\pm$ SEM, n=15-29, depending on day and treatment) [eggs (fem d)<sup>-1</sup>] of *Calanus helgolandicus* females feeding on P-limited and P-replete cultures of *Skeletonema marinoi* from outflow bottles of the chemostat set-up.159

Figure 5-9: Mean faecal pellet production rates (FPR) ( $\pm$ SEM, n=15-29 depending on day and treatment) [fp (fem d)<sup>-1</sup>] of *Calanus helgolandicus* females feeding on P-limited and P-replete cultures of *Skeletonema marinoi* from outflow bottles of the chemostat set-up.160

Figure 5-10: Mean hatching success ( $\pm$ SEM, n=13-29 depending on day and treatment) [%] of eggs produced by *Calanus helgolandicus* females feeding on P-limited and P-replete cultures of *Skeletonema marinoi* from outflow bottles of the chemostat set-up.161

Figure 5-11: Mean percentage of abnormal nauplii ( $\pm$ SEM, n=3-29 depending on day and treatment) [%] hatched from eggs produced by *Calanus helgolandicus* females feeding on P-limited and P-replete cultures of *Skeletonema marinoi* from outflow bottles of the chemostat set-up. .... 162

Figure 6-1: Mean cell concentrations ( $\pm$ SEM; n=3 except day 3: n=5, day 10: n=4) [ml<sup>-1</sup>] of *Pseudo-nitzschia delicatissima* during culture growth..... 172

Figure 6-2: Mean oxylipin production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for cells [pg cell<sup>-1</sup>] in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining). .... 173

Figure 6-3: Mean oxylipin production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for protein [ $\mu$ g (mg prot)<sup>-1</sup>] in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining). .... 174

Figure 6-4: Mean oxygen consumption rate (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for protein [ $\mu$ mol O<sub>2</sub> (mg prot min)<sup>-1</sup>] upon EPA-addition to cell lysate determined with the polarographic assay at pH=8.15 in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining). .... 175

Figure 6-5: Mean oxygen consumption rate (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for cells [fmol O<sub>2</sub> (cell min)<sup>-1</sup>] upon EPA-addition to cell lysate

determined with the polarographic assay at pH=8.15 in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining)..... 176

Figure 6-6: Oxygen consumption rate normalized for protein [ $\mu\text{mol O}_2$  (mg prot min) $^{-1}$ ] upon EPA-addition to cell lysate (n=1) determined with the polarographic assay in *Pseudo-nitzschia delicatissima* at pH=6.0 and 8.15..... 177

Figure 6-7: Mean fatty acid hydroperoxide (FAH) production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for cells [fmol cell $^{-1}$ ] determined with the colorimetric assay in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining). FAH production was defined as the difference in FAH concentrations between cell lysates after 20 min and blanks..... 178

Figure 6-8: Mean fatty acid hydroperoxide (FAH) production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for protein [ $\mu\text{mol}$  (mg prot) $^{-1}$ ] determined with the colorimetric assay in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining). FAH production was defined as the difference in FAH concentrations between cell lysates after 20 min and blanks..... 179

Figure 6-9: Mean production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for cells [pg cell $^{-1}$ ] of the oxo-acid (15-oxoacid), the epoxyalcohol (13,14-HEpETE) and the hydroxy-acid (15S-HEPE) in different growth phases of *Pseudo-nitzschia delicatissima*. ..... 180

Figure 6-10: The proposed 15S-lipoxygenase (LOX) pathway in *Pseudo-nitzschia delicatissima*. (EPA: Eicosapentaenoic acid, 15S-HpEPE: 15S-hydroperoxy eicosapentaenoic acid, HPL: hydroperoxide lyase, RED: hydroperoxide reductase, 15S-HEPE: hydroxy-eicosapentaenoic acid, ES: epoxyalcohol synthase, 13,14-HEpETE: epoxyalcohol)..... 182

Figure 7-1: Linear relationship of absorbance of cell lysate (655 nm) to *Skeletonema marinoi* pellet wet weight [mg] in the assay for protein determination. .... 195

Figure 7-2: Linear relationship of chlorophyll *a* amount [ng] to various amounts of *Skeletonema marinoi* pellet wet weight [mg]..... 196

Figure 7-3: Cell concentrations [cells ml<sup>-1</sup>] of *Skeletonema marinoi* in mesocosms B, C, D, E, and F during the course of the mesocosm experiment (days after inoculation). ..... 200

Figure 7-4: Mean concentrations (+SEM, n=2) of fatty acid hydroperoxide (FAH) normalized for protein [μmol (mg prot)<sup>-1</sup>] determined with the colorimetric assay in blanks (0 min), in cell lysate of a mesocosm sample (mesocosm F, day 10) (20 min), and in cell lysate upon EPA-addition (+EPA). ..... 201

Figure 7-5: Production of oxylipins other than aldehydes (n=1) normalized for cells [pg cell<sup>-1</sup>] in mesocosm bags C, E, and F during the course of the experiment (days after inoculation). ..... 202

Figure 7-6: Production of aldehydes and oxylipins other than aldehydes (n=1) normalized for cells [fmol cell<sup>-1</sup>] in mesocosm bags C, E, and F during the course of the experiment (days after inoculation). ..... 203

Figure 7-7: Concentration of phosphate [μg l<sup>-1</sup>] (n=1) in mesocosm bags C, E, and F during the course of the experiment (days after inoculation; surrounding sea measured as control). ..... 205

Figure 7-8: Concentration of nitrate [μg l<sup>-1</sup>] (n=1) in mesocosm bags C, E, and F during the course of the experiment (days after inoculation; surrounding sea measured as control). 205

Figure 7-9: Concentration of silicate [μg l<sup>-1</sup>] (n=1) in mesocosm bags C, E, and F during the course of the experiment (days after inoculation; surrounding sea measured as control). ..... 206

Figure 7-10: Production of aldehydes and oxylipins other than aldehydes (n=1) normalized for chlorophyll *a* [nmol (μg chl *a*)<sup>-1</sup>] in mesocosm bags C, E, and F during the course of the experiment (days after inoculation). ..... 207

Figure 7-11: Production of aldehydes and oxylipins other than aldehydes (n=1) normalized for carbon [nmol (mg C)<sup>-1</sup>] in mesocosm bags C, E, and F during the course of the experiment (days after inoculation). ..... 208

Figure 7-12: Mean faecal pellet production rates (FPR) ( $\pm$ SEM, n=12-15 depending on day and treatment) [ $\text{fp (fem d)}^{-1}$ ] of *Calanus finmarchicus* females collected from mesocosm bags B, C, and F during the course of the experiment (days after inoculation). ..... 209

Figure 7-13: Mean hatching success of eggs ( $\pm$ SEM; n=6-15 depending on day and treatment) [%] produced from *Calanus finmarchicus* females collected in mesocosm bags B, C, and F during the course of the experiment (days after inoculation). ..... 210

Figure 7-14: Mean percentage of abnormal nauplii ( $\pm$ SEM; n=8-15 depending on day and treatment) [%] hatched from eggs produced by *Calanus finmarchicus* females collected from mesocosm bags B, C, and F during the course of the experiment (days after inoculation). ..... 211

Figure 7-15: Nauplius of *Calanus finmarchicus* from the mesocosm experiment stained positive for apoptosis with TUNEL. .... 212

Figure 7-16: Mean concentrations ( $\pm$ SEM, n=2) of fatty acid hydroperoxide (FAH) normalized for protein [ $\mu\text{mol (mg prot)}^{-1}$ ] determined with the colorimetric assay in blanks (0 min), in cell lysate of the *Skeletonema marinoi* strain used for inoculation of the mesocosms, cultured in Bergen and at SZN (20 min), and in cell lysate upon EPA-addition (+EPA). ..... 213

Figure 7-17: Mean oxygen consumption rate (n=1) [ $\mu\text{mol O}_2 \text{ (mg prot min)}^{-1}$ ] of cell lysate (lysate) and cell lysate upon EPA-addition (lysate+EPA) in both replicates of the mesocosm strain of *Skeletonema marinoi* cultured at SZN determined at pH=8.15 in the polarographic assay. .... 214

Figure 7-18: Production of aldehydes and oxylipins other than aldehydes ( $\pm$ SEM, n=2) normalized for cells [ $\text{fg cell}^{-1}$ ] in the mesocosm strain of *Skeletonema marinoi* cultured at SZN and in Bergen. .... 215

# 1 Introduction

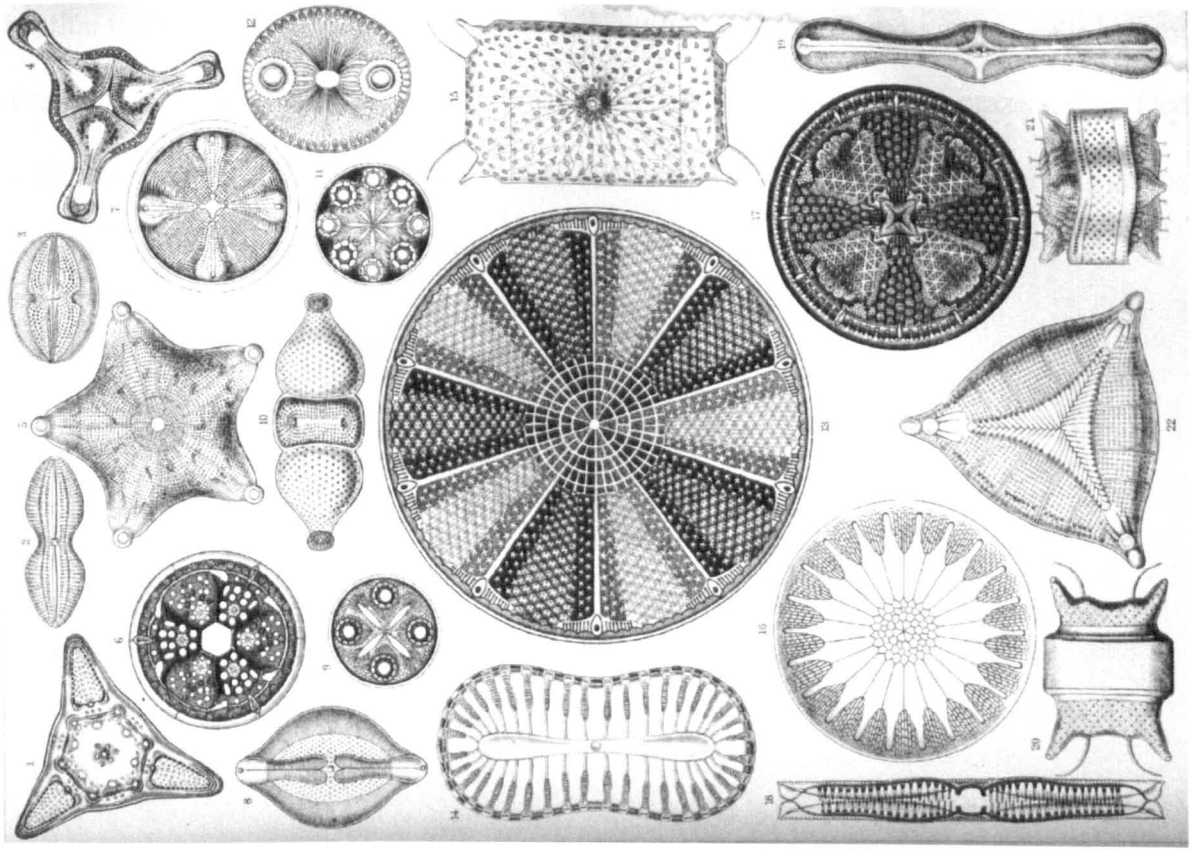
## 1.1 Plankton in the Marine Environment

At first glance, the open ocean appears a homogeneous environment and it seems surprising that such a system could be capable of supporting productive and diverse ecosystems. Yet, small-scale variations created by the dynamic fluctuations of abiotic and biotic factors support high productivity, as well as biodiversity. Important players in the open water column, the so called pelagic realm, are planktonic organisms. They form a group of drifting organisms whose movements cannot compete with large-scale movements of the water column and they are therefore subject to the ocean currents. As in terrestrial systems, plankton communities are characterized by primary producers, autotrophic phytoplankton, and primary consumers, heterotrophic zooplankton, as well as higher trophic levels, such as fish or marine mammals. Carbon fixation of phytoplankton has been judged to equal that of terrestrial systems, contributing ~50% to total global primary production (Field *et al.* 1998).

The phytoplankton is composed mainly of unicellular eukaryotic algae which range in size from ~2-200  $\mu\text{m}$  in diameter and exist in a great variety of shapes and sizes. These microalgae include green algae, coccolithophorids, diatoms and dinoflagellates, even though many species of the latter phylum are hetero- or mixotrophic. Recently, the importance of even smaller photosynthetic prokaryotic organisms has been recognized. These abundant cyanobacteria belong to the genus *Synechococcus* and *Prochlorococcus* and contribute fundamentally to primary productivity in many areas of the world's oceans (Azam and Worden 2004).

Diatoms often form blooms in coastal and upwelling areas and can contribute up to 50% to total marine primary production (Mann 1999). They are also highly abundant in freshwater systems and can be found as epiphytes, forming biofilms on biological surfaces and sediments. The characteristic diatom “frustule”, the strong silica cell wall, makes diatoms important not only in the global carbon cycle, but also in the global silica cycle (Treguer *et al.* 1995). Massive sink-outs from diatom blooms are of geological importance as they form siliceous oozes in the deep sea (Smetacek 1985). Apart from its role in sinking, the diatom frustule has been suggested to function as a protection against zooplankton grazers (Hamm *et al.* 2003) because of its flexibility and robustness, for which it finds applications in nanotechnology. The highly intricate design and diversity of the diatom frustule also conveys a characteristic beauty to this class of microalgae (Figure 1-1).





**Figure 1-1: Diatoms from Ernst Haeckel’s “Kunstformen der Natur” (1904).**

Zooplankton feed on diatoms and other phytoplankton and make up the animal part of the plankton. These heterotrophic organisms include protozoa, such as foraminiferans, radiolarians, and heterotrophic dinoflagellates, and metazoa, such as cnidarians, crustaceans, chaetognaths, molluscs, and chordates. An important group of zooplankters are the calanoid copepods, small crustaceans with a size of 0.5-2 mm. Their name “copepoda” derives from the Greek word “kope”, meaning “oar” and “podos”, meaning “foot” (Mauchline 1998). In fact, copepods are characterized by paddle-shaped appendages that function as filters for collecting food from the environment, even though many species are also raptorial. Copepods also show a variety of shapes and sizes (Figure 1-2) and are abundant in marine and freshwater systems, occurring in the pelagic, as well as in the

benthic realm. They can contribute up to 70% to the total plankton biomass, with highest abundances found in the polar region (Longhurst 1985). As herbivorous grazers, they feed willingly on diatoms (Paffenhoefer 1976; Paffenhoefer 2002) and have evolved strong teeth-like structures to break open the diatom frustule (Sullivan *et al.* 1975).

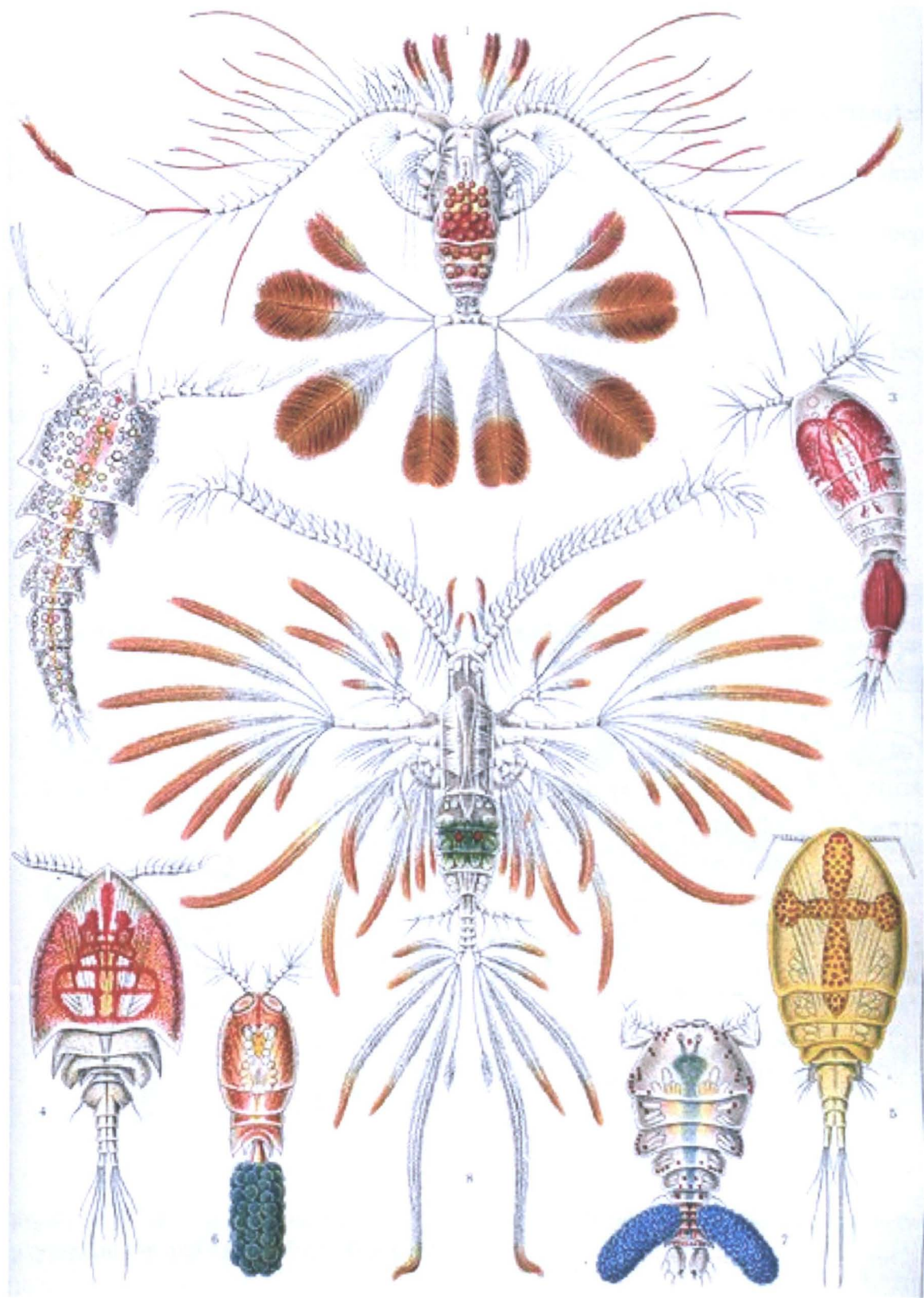


Figure 1-2: Copepods from Ernst Haeckel's "Kunstformen der Natur" (1904).

## 1.2 Diatom-copepod Interactions

Traditionally, the diatom-copepod link has been considered fundamental for the transfer of energy from primary to secondary production and further up the food web to sustainable fisheries (Runge 1988; Cushing 1989; Mann 1993). Fish larvae depend greatly on copepod eggs and nauplii (Mann 1993), making copepod secondary production an important factor in the flow of energy and carbon from primary production to higher trophic levels (Figure 1-3).

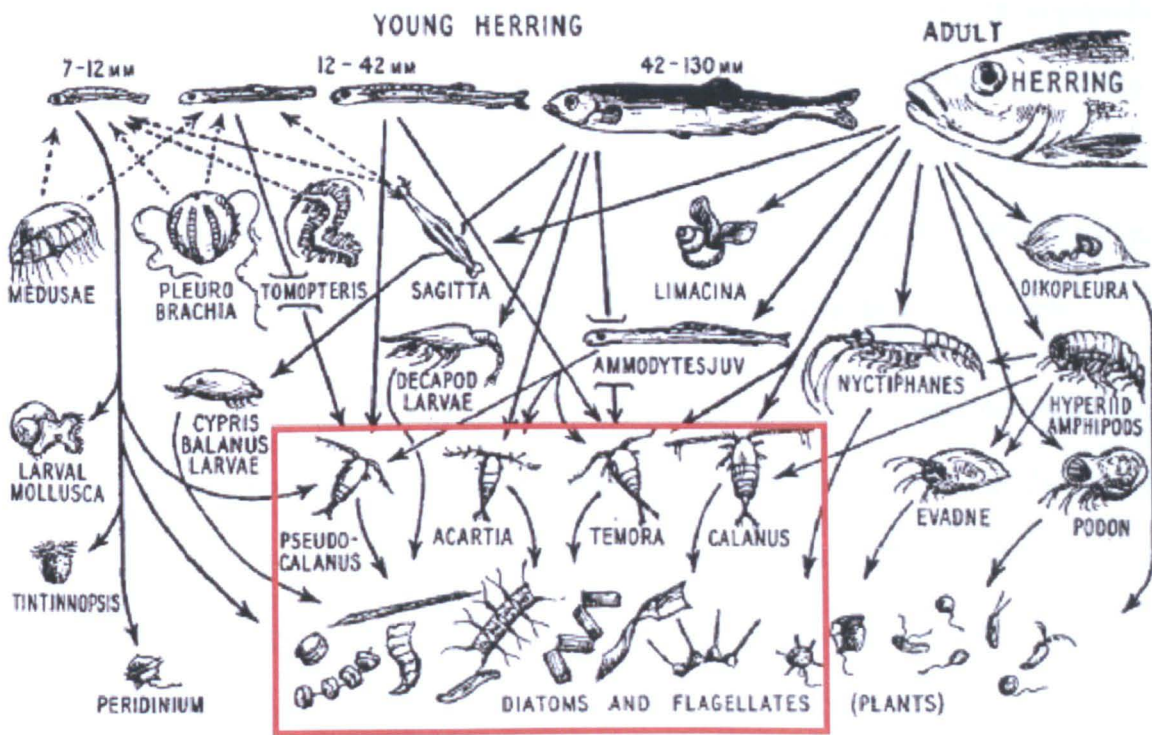


Figure 1-3: The classical marine food web; the box highlights the interactions between phytoplankton and copepods (Hardy 1959).

### 1.2.1 Copepod Reproduction

Egg production rates are usually considered a good indicator of secondary production in copepods (Kimmerer *et al.* 2005). Egg production rates in turn are usually positively correlated with food supply and thereby are highest during blooms (Beckman and Peterson 1986; Peterson and Kimmerer 1994; Kimmerer *et al.* 2005). However, the development of copepod eggs to hatched nauplii and the following development of nauplii to adult copepods are just as important as initial egg production rates for controlling cohort size of the next generation. Important aspects for determining copepod secondary production are therefore factors controlling egg mortality, for example grazing on the produced eggs (Kiorboe *et al.* 1988). Cannibalism on copepod eggs has been observed in copepods (Landry 1978), as well as grazing by other zooplankton (Beckman and Peterson 1986). Physical factors such as sinking (e. g. through formation of resting eggs) (Uye 1982) or biological factors such as diseases (Peterson and Kimmerer 1994) or parasites e. g. dinoflagellates (Drebes 1978), may all play an important role in copepod recruitment.

Ianora and Poulet (1993) first observed that hatching success of copepod eggs can furthermore be compromised when females feed on certain diatom diets, in their case *Thalassiosira rotula*. Over the past 15 years, doubts have arisen as to the beneficial role of diatoms for copepod reproduction. Whereas reduced hatching success of copepod eggs was previously ascribed to a lack of fertilization (Katona 1975), Ianora *et al.* (1992) demonstrated that reduced egg viability could be found even in fertilized eggs and that reduced hatching success was due to maternal diets of certain diatom species (Ianora and Poulet 1993; Ianora *et al.* 1995). In fact, in a world-wide survey conducted by Ban *et al.* (1997), only one out of the 17 diatom species studied did not compromise copepod reproductive success. In addition to reducing hatching success of eggs, maternal diets of



certain diatom species lead to malformations in hatched nauplii such as missing or reduced limbs (Poulet *et al.* 1995; Ianora *et al.* 2004). The reason for these malformations has been identified as the occurrence of apoptotic tissue in nauplii (Poulet *et al.* 2003; Romano *et al.* 2003), leading ultimately to reduced hatching of impacted eggs. Although in some cases egg production is also compromised by diatoms (Ban *et al.* 1997), often these microalgae support high egg production rates (Ianora and Poulet 1993; Miralto *et al.* 2003). The main reproductive parameters impaired by maternal diatom diets are hatching success and naupliar development (Poulet *et al.* 1995; Ianora *et al.* 2004). This discovery adds another important facet to copepod recruitment and needs to be taken into account when examining copepod population dynamics.

### **1.2.2 Detrimental Impact of Diatom Diets**

The contribution of diatoms to copepod diets had been questioned previously because of the recognized importance of alternative diets in an increasingly complex food web model (Kleppel *et al.* 1991). These authors suggested that copepods may graze preferentially on dinoflagellates or microzooplankton and that the role of diatoms in copepod nutrition had been given too much importance. The nutritional aspect of copepod secondary production, especially the quality of food supply, was highlighted by these authors and the hypothesis formed that a mixed and varied diet should result in highest egg production rates (Kleppel 1993). This in turn emphasised the importance of non-diatom foods in copepod diets. Contemporarily, results obtained by Ianora and Poulet (1993) demonstrated a deleterious effect of diatom diets on reproductive fitness. One of the explanations proposed for the deleterious effect of diatoms on copepod reproductive success was a nutritional deficiency in diatoms (Jonasdottir and Kiorboe 1996). Certain dietary components including

polyunsaturated fatty acids (PUFAs) have been judged fundamental for egg production, as well as for hatching success of copepod eggs (Mueller-Navarra *et al.* 2000; Jonasdottir *et al.* 2002; Arendt *et al.* 2005). However, the PUFA-content in diatoms tends to be high (Berge *et al.* 1995), so a potential nutritional deficit in diatoms is probably due to some other component. Furthermore, several authors failed to find a correlation between naupliar development and nutritional factors, such as nitrogen to carbon ratios, PUFA-, or sterol-content of phytoplankton food (Dutz *et al.* 2008; Koski *et al.* 2008). It has also been suggested that fatty acids from diatom cells are rapidly degraded upon grazing, making them unavailable for copepod metabolism and thereby inducing PUFA-deficiency (Wichard *et al.* 2007).

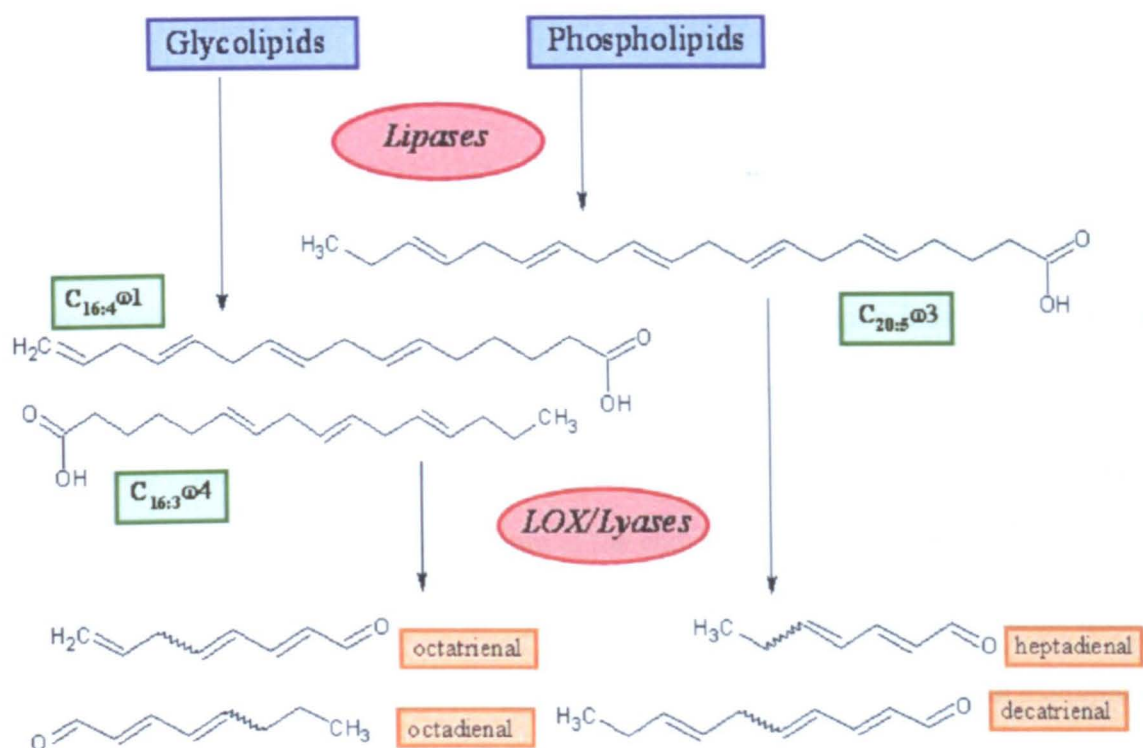
As diatoms do not seem to be lacking an obvious nutritional factor (Ianora and Poulet 1993), an alternative explanation for the detrimental effect of diatoms on copepods concentrated on the production of anti-mitotic compounds by this class of microalgae, held responsible for compromising the development of copepod embryos (Poulet *et al.* 1994). This hypothesis was based on incubation experiments of copepod eggs with diatom extracts which resulted in compromised egg development (Poulet *et al.* 1994; Uye 1996). Production of toxic compounds was confirmed by Miralto *et al.* (1999), who identified polyunsaturated aldehydes (PUAs) as the anti-mitotic agents in diatoms. The first anti-mitotic compounds isolated by these authors from marine diatoms were two isomers of decatrienal and one isomer of decadienal. Both belong to the group of  $\alpha,\beta,\gamma,\delta$ -unsaturated aldehydes characterized by a 2,4-pentadiene moiety conjugated to the aldehydic group (Pohnert 2002). These and other shorter-chain PUAs had been previously described in the freshwater diatoms *Melosira varians* and *Fragilaria sp.*, but had not been investigated for their effect on grazer secondary production (Wendel and Juettner 1996). Miralto *et al.* (1999) identified them for the first time in the marine environment and

correlated them to a decrease in reproductive success of calanoid copepods. Since then, PUAs have been discovered in several marine and freshwater species (Wichard *et al.* 2005). Also the shorter-chain PUAs, namely octadienal and octatrienal, as well as heptadienal have been isolated from marine species (d'Ippolito *et al.* 2002a), even though greater biological activity has been observed for longer-chain homologues (d'Ippolito *et al.* 2002b; Adolph *et al.* 2003; Ceballos and Ianora 2003). This type of insidious mechanism for impairing grazers by interfering with reproduction is new in the marine environment, because most grazer defences act directly, e. g. by grazer deterrence or poisoning.

### **1.2.3 Production of Polyunsaturated Aldehydes by Diatoms**

Polyunsaturated aldehydes (PUAs) result from the oxidation of polyunsaturated fatty acids (PUFAs) and therefore belong to the class of oxylipins (Figure 1-4). The term “oxylipins” was introduced by Gerwick *et al.* (1991) for describing fatty acid derived oxygenated compounds in marine algae. Oxylipins were defined by these authors as: “oxygenated compounds which are formed from fatty acids by reactions involving at least one step of mono- or dioxygenase-catalyzed oxygenation” (Gerwick *et al.* 1991). This definition thereby includes the well-known mammalian eicosanoids, as well as “biosynthetically related compounds of longer and shorter chain length” (Gerwick *et al.* 1991) produced by algae, plants, and fungi.





**Figure 1-4: Production of the polyunsaturated aldehydes octatrienal and octadienal from  $C_{16}$ -fatty acids released from glycolipids, and production of heptadienal and decatrienal from  $C_{20}$ -fatty acids released from phospholipids; adapted from d'Ippolito *et al.* (2004) and Pohnert (2005).**

In diatoms, PUAs are rapidly released upon loss of cell integrity, for example upon grazing by copepods (Pohnert 2000). Upon wounding of the cell, lipases release PUFAs from complex lipids (Figure 1-4). Chloroplastic glycolipids are a source of  $C_{16}$ -PUFAs, such as  $C_{16:4}\omega 1$  and  $C_{16:3}\omega 4$  which are converted to the  $C_8$ -aldehydes octatrienal and octadienal, respectively (d'Ippolito *et al.* 2003; d'Ippolito *et al.* 2004; Cutignano *et al.* 2006) (Figure 1-4). The  $C_{20}$ -PUFA eicosapentaenoic acid ( $C_{20:5}\omega 3$ ) is released from membrane phospholipids and serves as a precursor for the  $C_7$ -aldehyde heptadienal and the  $C_{10}$ -aldehyde decatrienal (Pohnert 2002; d'Ippolito *et al.* 2003; d'Ippolito *et al.* 2004) (Figure 1-4). The PUFAs released by lipases are oxidized by lipoxygenases (LOXs) to

fatty acid hydroperoxides (FAHs) which are converted to PUAs by lyase activity further down-stream (Pohnert 2002; d'Ippolito *et al.* 2006) (Figure 1-4). Therefore LOX enzymes play an important role in the production of oxylipins from fatty acids because they are responsible for the first oxidative step. The reason behind rapid production of oxylipins upon cell rupture probably lies in the compartmentalization of enzymes and substrates, allowing release of free fatty acids as substrate for LOX only upon loss of cell integrity (Pohnert 2005). Although the release of PUAs had been previously excluded for intact cells (Pohnert 2002), it has been recently postulated that intact cells of *Skeletonema marinoi* release PUAs into the cell medium at the end of stationary phase before going into senescence (Vidoudez and Pohnert 2008). This release of PUAs by intact cells in specific growth phases has been interpreted as a regulatory mechanism and given as an example for the possible role of aldehydes in cell to cell signalling (Vardi *et al.* 2006). Another alternative function, apart from grazer defence and cell to cell signalling proposed for PUAs in marine systems is allelopathy. PUAs have been described to impact phytoplankton (Casotti and Mazza 2005; Ribalet *et al.* 2007a), as well as bacteria (Ribalet *et al.* 2008), possibly imparting a competitive advantage to PUA-producing species.

#### **1.2.4 New Insights into Diatom Oxylin Metabolism**

The proposed role of polyunsaturated aldehydes (PUAs) as an insidious defence mechanism against grazers by induction of a teratogenic effect has been criticized (Jonasdottir *et al.* 1998; Irigoien *et al.* 2002) and its relevance in natural systems is still debated (Sommer 2009). Criticisms have been founded mainly on contradictory results obtained from field studies, which sometimes failed to find a deleterious effect of diatoms on copepod reproduction (Pond *et al.* 1996; Irigoien *et al.* 2000; Irigoien *et al.* 2002;

Verheye and Irigoien 2002; Sommer 2009). However, numerous studies have also validated the negative effect of phytoplankton assemblages on copepod secondary production during periods of high diatom concentrations, even though effects could not always be correlated to the production of PUAs by phytoplankton (Laabir *et al.* 1995; Ianora *et al.* 2004; Poulet *et al.* 2007; Ianora *et al.* 2008).

A possible explanation for the lack of correlation between PUA production by diatoms and copepod reproductive success may be that PUAs are not the only active molecules produced by diatoms. Recently, other oxylipins such as hydroxy-acids and epoxyalcohols have been isolated (d'Ippolito *et al.* 2005; Fontana *et al.* 2007b), which also stem from the oxidative metabolism of fatty acids (Figures 1-5 and 1-6). These molecules have also been found to negatively impact copepod reproductive success (Fontana *et al.* 2007b; Ianora *et al.* 2008) and even the intermediate fatty acid hydroperoxides (FAHs) have been found to reduce hatching success when FAHs are incubated directly with copepod eggs (Fontana *et al.* 2007b). These new insights into oxylipin metabolism in diatoms and the discovery of new “toxic” metabolites may explain the deleterious effect of diatoms on hatching success in the absence of PUA-production (Ianora *et al.* 2008).

Oxylipin metabolism in diatoms is quite complex, with variations found among strains of the same species (Pohnert *et al.* 2002; Taylor *et al.* 2009) and even among different physiological status of the same diatom culture due to e. g. different nutrient regimes (Ribalet *et al.* 2007b; Ribalet *et al.* 2009) or various growth phases (Ribalet *et al.* 2007b; d'Ippolito *et al.* 2009). This great plasticity in oxylipin metabolism may shed some light on the often contradictory results obtained in the field. In addition to the great variability in diatom oxylipin metabolism observed on all levels, different species of copepods also display varying responses to oxylipin-producing diatoms (Paffenhoefer *et al.* 2005).

Copepods evidently vary in their sensitivity towards oxylipins and certain copepod species may be more apt at dealing with oxidative stress than others, due to detoxification mechanisms present in these species (Ianora 2005; Lee *et al.* 2007; Souza *et al.* 2007). The variability in oxylipin production by diatoms on one hand and the flexible response of consumer copepods on the other, may explain the lack of effect described in some cases in the literature. It has additionally been suggested that diatoms may be critical only during a short time window (Sommer 2009). As diatoms can successfully support somatic growth of copepods, alternative diets may only be important during the limited time period of reproduction (Sommer 2009), reducing the impact of diatoms on copepod reproduction in the field.

The increasing number of studies on diatom-copepod interactions, especially under the aspect of diatom oxylipin metabolism is creating a complex picture with conclusions depending on the diatom and copepod species examined (Ban *et al.* 1997), as well as on other contextual factors such as alternative food sources (Sommer 2009). While undoubtedly some diatom species deleteriously impact the reproduction of some copepod species, there is still considerable on-going debate as to what extent the results observed in the laboratory with mono-cultures are relevant to the field, where food supply is diverse (Sommer 2009).

### 1.3 The Role of Lipxygenases in Oxylipin Production

The variations in the production of oxylipins by different diatom species are presumably due to different arrays of enzymatic systems. As mentioned above, oxylipin production is initiated by the oxidation of fatty acids by lipxygenase (LOX) to fatty acid

hydroperoxides (FAHs) (Pohnert 2002). Therefore LOX is one of the key enzymes in the production of oxylipins, even though it has been suggested that the release of fatty acids by lipases could also be a rate-determining step in the reaction (d'Ippolito *et al.* 2003).

### **1.3.1 General Functions of Lipoxygenases**

Lipoxygenases (LOXs) are found in almost all eukaryotes, where they are responsible for the dioxygenation of fatty acids by means of a co-factor, a non-heme iron contained in the reactive site, which needs to be in the oxidized ( $\text{Fe}^{3+}$ ) state to be active (Schilstra *et al.* 1994). LOXs are fundamental for cell functioning, being involved in signalling pathways and in the induction of structural and metabolic changes in cells (Brash 1999). Signalling functions result from the production of fatty acid hydroperoxides (FAHs) from polyunsaturated fatty acids (PUFAs), which can be highly specific (Brash 1999). FAHs can act directly as signalling molecules, for example in a reduced form as a hydroxy-acid (Piomelli *et al.* 1987) or be converted further into secondary oxylipins with signalling functions, as in the production of leukotrienes in mammals (Yamamoto *et al.* 1997) or jasmonates in plants (Royo *et al.* 1996; Feussner and Wasternack 2002). However, LOX can also directly oxidize esterified fatty acids, inducing changes in membrane structure and provoke a more general lipid peroxidation (Feinmark and Cornicelli 1997; Kuehn *et al.* 2002). Lastly, through oxidation of esterified fatty acids, they can also be involved in the liberation of fatty acids for metabolic purposes, facilitating secondary oxidation of membrane-bound FAHs to liberate fatty acids from lipid stores (Feussner *et al.* 1997). Plants and animals differ in their substrate for LOX activity with  $\text{C}_{18}$ -fatty acids such as linoleate and  $\alpha$ -linolenate acids mainly used by plant LOX (Feussner and Wasternack

2002) and C<sub>20</sub>-fatty acids such as arachidonic and eicosapentaenoic acids, as well as C<sub>22</sub>-fatty acids such as docosahexaenoic acid, by animal LOX (Kuehn *et al.* 2002).

In plants, oxylipins produced by LOX pathways are involved in regulating growth and development (Miyamoto *et al.* 1997), such as germination of seedlings (Ohta *et al.* 1986; Melan *et al.* 1994), tuberisation and flowering (Creelman and Mullet 1995), and ripening of fruit (Ferrie *et al.* 1994; Kausch and Handa 1997), as well as in senescence (Yamane *et al.* 1981; Sembdner and Parthier 1993). Oxylipins are furthermore involved in induced defence reactions against pathogens, as well as in wound responses. LOX enzymes in plants oxidize C<sub>18</sub>-fatty acids either at the 9- or 13-position of the carbon backbone (9- or 13-LOX, respectively) (Feussner and Wasternack 2002). Further conversions of the produced hydroperoxides lead to a plethora of oxylipin molecules with the above-mentioned multitude of functions. Lyase activity, for example, leads to the production of volatile C<sub>6</sub>- or C<sub>9</sub>-aldehydes which give plants their characteristic “leafy odour” and may be involved in plant-plant communications, as well as having antimicrobial and antifungal properties (Matsui *et al.* 2000). 13-Hydroperoxides may also be converted to jasmonates, cyclic C<sub>12</sub>-compounds involved in plant signalling and induced defence responses, such as the expression of defence genes upon herbivore attack (Farmer and Ryan 1990; Wasternak and Parthier 1997). Additionally, the reduction of hydroperoxides to the corresponding alcohols may be involved in wound healing, as they are structural components of the cuticle (Blee 1998).

In animals, 5-, 12-, and 15-LOX pathways have been described (Serhan 1997). These lead to the production of oxylipins mainly from arachidonic acid (C<sub>20:4</sub>ω6) and play important roles in inflammatory and immune responses (Samuelsson 1983). These oxylipins can be divided into two major groups, the prostanoids which include prostaglandins,

prostaglandins, and thromboxanes, and the leukotrienes (Samuelsson 1980). The former are produced by cyclooxygenases which carry out two dioxygenation reactions resulting in the formation of a 5-carbon ring, whereas the latter are the products of 5-LOX activity (Needleman *et al.* 1986). Recently, other oxylipins resulting also from the oxidation of arachidonic acid by LOX pathways have been identified, such as hepoxilins from 12-LOXs (Pace-Asciak and Martin 1984) and lipoxins, which seem to result from multiple LOX pathways and are anti-inflammatory mediators (Mitchell *et al.* 2002). Other lipid-derived mediators involved in anti-inflammatory reactions and in resolution of inflammation derive from  $\omega$ 3-PUFAs, such as the C<sub>20</sub>-PUFA eicosapentaenoic acid or the C<sub>22</sub>-PUFA docosahexaenoic acid (DHA). These so called resolvins are produced by cyclooxygenases and form a class of polyhydroxy-fatty acids (Serhan *et al.* 2002). DHA is also the precursor for docosatrienes, hydroxy-containing docosanoids which are characterized by conjugated triene structures (Serhan 2005) and derive from epoxide-containing intermediates (Hong *et al.* 2003). Akin to the resolvins, these oxylipins act as endogenous protective mediators in inflammatory reactions (Hong *et al.* 2003).

### 1.3.2 Lipoxygenase Activity in Diatoms

Lipoxygenase (LOX) metabolism has some interesting aspects in diatoms. In contrast to plants, C<sub>18</sub>-fatty acids are not preferred substrates for diatom LOX (Pohnert 2000; d'Ippolito *et al.* 2006) and are present only in trace amounts in these microalgae (d'Ippolito *et al.* 2003). Instead, LOX enzymes in diatoms, akin to those in mammals, metabolize the C<sub>20</sub>-fatty acids eicosapentaenoic acid (EPA) and arachidonic acid, even though the latter is probably not relevant under natural conditions, due to its low presence in diatoms (Pohnert 2002; d'Ippolito *et al.* 2006). In addition to oxidizing EPA (Figure 1-6), diatom oxidative

fatty acid metabolism demonstrates a novel LOX metabolism on C<sub>16</sub>-fatty acids (C<sub>16:3</sub> and C<sub>16:4</sub>) (d'Ippolito *et al.* 2003) (Figure 1-5). Several classes of LOXs have been identified in diatoms, differing in positional specificity and in the stereochemistry of the produced fatty acid hydroperoxides (FAHs) (Figures 1-5 and 1-6) and most diatoms demonstrate more than one apparent LOX activity. The complexity in LOX enzymes explains some of the diverse oxylipin profiles identified in different diatom species. Diversity is further increased by down-stream enzymes involved in transforming FAHs to secondary oxylipins. Also in this field, diatoms offer a possibility for discovery of novel enzymes, such as a novel hydroperoxide halolyase in *Stephanopyxis turris* (Wichard and Pohnert 2006). Hydroperoxide lyases (HPLs) are generally assumed responsible for transforming FAHs into aldehydes (Figures 1-5 and 1-6) (Andreou *et al.* 2009), although a bifunctional LOX activity has also been suggested which may directly transform PUFAs into polyunsaturated aldehydes (PUAs) (Pohnert 2005). Figures 1-5 and 1-6 give a selective overview of the LOX activities on C<sub>16</sub>- and C<sub>20</sub>-fatty acid precursors, respectively, relevant for *Thalassiosira rotula*, *Skeletonema marinoi*, and *Chaetoceros affinis*.



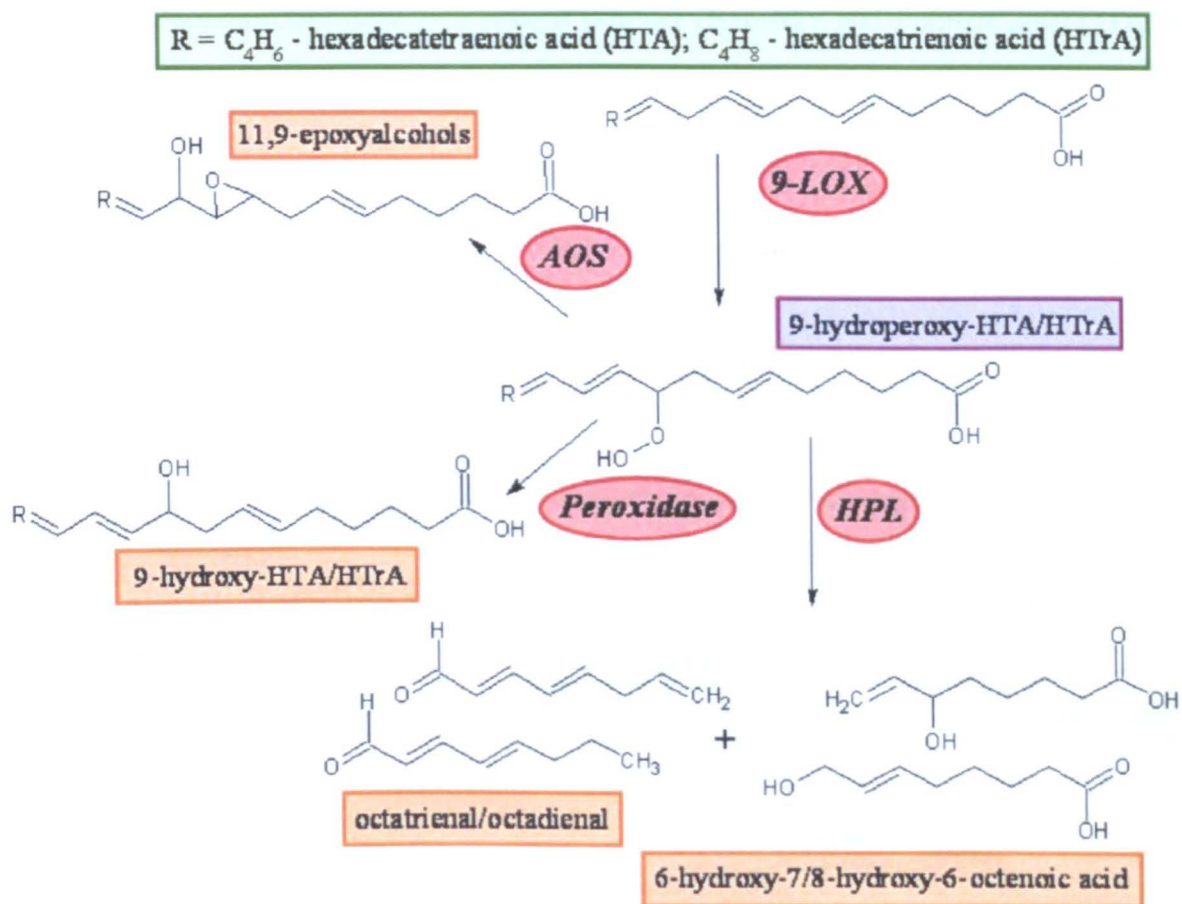
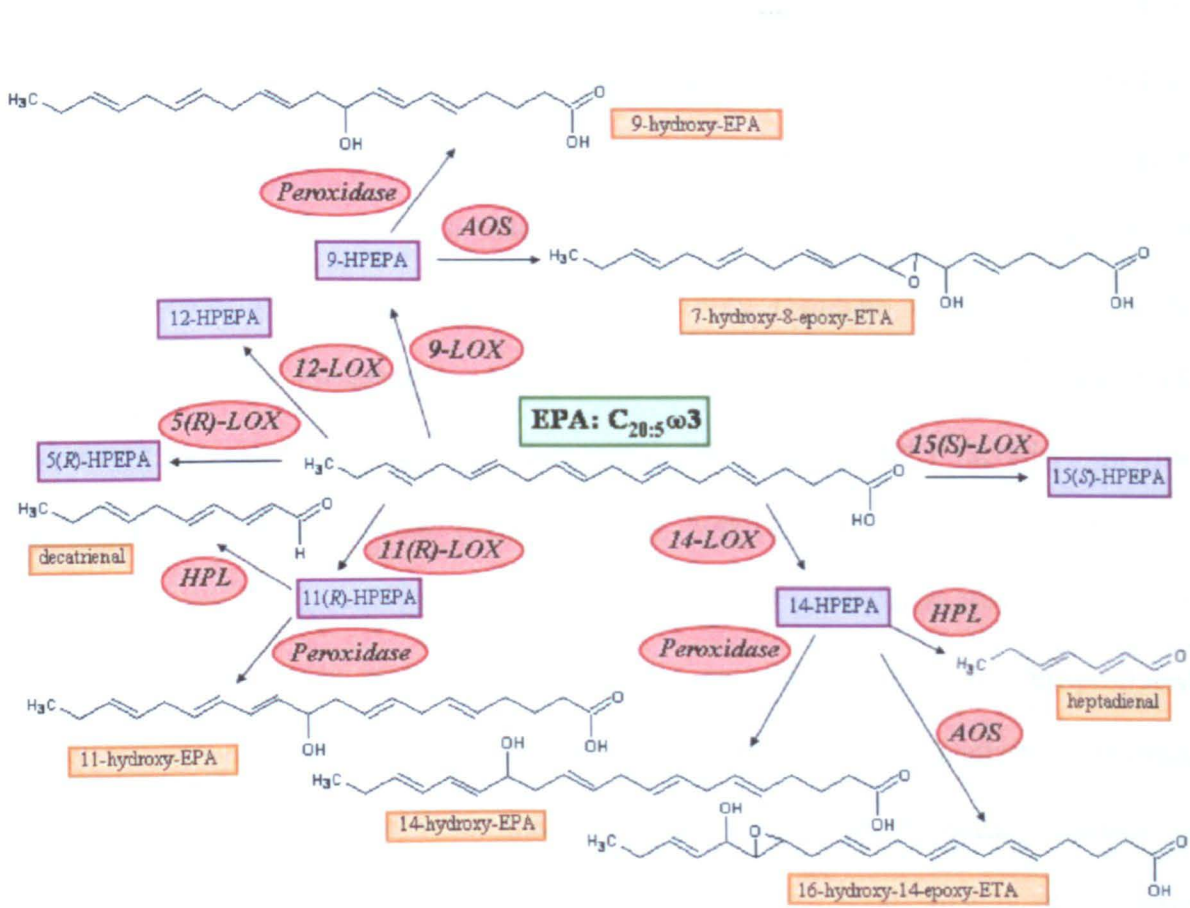


Figure 1-5: 9-lipoxygenase (LOX) activity on C<sub>16</sub>-fatty acids (HTA, HTrA) in diatoms; adapted from Barofsky and Pohnert (2007) and Andreou *et al.* (2009) (AOS: allene oxide synthase, HPL: hydroperoxide lyase).

*T. rotula* and *S. marinoi* have been found to both possess 9(*S*)-LOX activity for oxidizing the C<sub>16</sub>-fatty acids hexadecatetraenoic acid (HTA) and hexadecatrienoic acid (HTrA) to the respective hydroperoxides (Figure 1-5) (d'Ippolito *et al.* 2006; Fontana *et al.* 2007b). These can then be further converted by a presumable allene oxide synthase (AOS) to the corresponding epoxyalcohols (Figure 1-5). Alternatively, the 9(*S*)-hydroperoxides can be either reduced to the corresponding hydroxy-acids via a presumable peroxidase activity or converted to aldehydes (Figure 1-5). Production of aldehydes seems to follow a unique hydroperoxide lyase (HPL) pathway in *T. rotula*, leading to the production of octatrienal in the case of HTA and octadienal in the case of HTrA, together with the production of a

short-chain hydroxy-fatty acid as a second product (Barofsky and Pohnert 2007) (Figure 1-5).



**Figure 1-6:** Selective overview of oxylipin metabolism of the C<sub>20</sub>-fatty acid eicosapentaenoic acid (EPA) in diatoms; adapted from Andreou *et al.* (2009) (LOX: lipoxygenase, AOS: allene oxide synthase, HPL: hydroperoxide lyase, HPEPA: hydroperoxy-eicosapentaenoic acid).

The metabolism of C<sub>20</sub>-fatty acids is more complex with several different LOX activities leading to the production of hydroxy-acids, epoxyalcohols, and aldehydes (Figure 1-6). EPA is presumably oxidized at the 9-carbon atom similar to the C<sub>16</sub>-fatty acids (Figure 1-5). The intermediate 9-hydroperoxide (9-HPEPA) is transformed either to 7-hydroxy-8-epoxy-eicosatetraenoic acid (epoxyalcohol) by a presumable AOS activity or

to the hydroxy-fatty acid (9-hydroxy-EPA) via a reducing activity, possibly carried out by peroxidases (Figure 1-6). However, numerous other LOX activities have been described for C<sub>20</sub>-fatty acids, such as a 15(*S*)-, as well as a 5(*R*)-activity, in *S. marinoi* (Fontana *et al.* 2007b) (Figure 1-6). 14-LOX activity on EPA is assumed to lead to the formation of heptadienal through lyase (HPL) activity on the intermediate hydroperoxide (14-HPEPA) in *S. marinoi* (previously *S. costatum* (Sarno *et al.* 2005)) (d'Ippolito *et al.* 2004) (Figure 1-6). Alternative pathways may lead from the 14-HPEPA to the production of 14-hydroxy-EPA in *C. affinis* via peroxidase activity (Fontana *et al.* 2007b) or to epoxyalcohols via a presumable AOS (Figure 1-6). An 11(*R*)-LOX activity has been described for the production of decatrienal via lyase (HPL) activity on the 11(*R*)-hydroperoxide in *T. rotula* (d'Ippolito *et al.* 2006) (Figure 1-6). Lastly, a 12-LOX activity has been reported for the diatom *S. turris* (Wichard and Pohnert 2006). The variety of LOX activities found in diatoms coupled to various enzymatic activities on the produced hydroperoxides, such as HPL, AOS, and reducing activities produce a plethora of oxylipin molecules. Diatom oxylipins other than aldehydes may serve the same functions as proposed for aldehydes, such as grazer defence, cell signalling, or allelopathy, or may have novel functions that still remain to be discovered and described.

## 1.4 Aims of the Thesis

The deleterious effect of diatoms on copepod reproduction due to oxidative fatty acid metabolism is usually analysed by quantification of oxylipin production via mass spectrometry after extraction of phytoplankton samples with organic solvents. This method is precise and sensitive, but involves a lengthy procedure and requires the necessary equipment, as well as the know-how to interpret the spectrometric data. Therefore the need

for a more rapid and easy method to quantify these compounds has emerged, such as a colorimetric assay for the analysis of lipoxygenase (LOX) activity (Fontana *et al.* 2007b), the enzyme responsible for the first oxidative step in the production of oxylipins by oxidizing fatty acids to fatty acid hydroperoxides (FAHs) (Pohnert 2002). Hence the object of this thesis was to develop such a method for quantifying oxylipin production in diatoms. Furthermore, various aspects influencing LOX activity were examined, as well as their effect on the reproductive success of calanoid copepods. Analysis of aldehydes and other oxylipins was carried out in close collaboration with the group of Angelo Fontana (ICB-CNR, Pozzuoli).

1) The first part of the thesis consisted of analyzing field phytoplankton samples, collected during the *Skeletonema marinoi*-dominated spring bloom in the Northern Adriatic Sea in 2005, for oxylipin production and LOX activity by the colorimetric assay. This provided an ample data pool for examining whether oxylipin production was correlated to LOX activity. It furthermore allowed an evaluation of whether hatching success of eggs produced by *Calanus helgolandicus* during the same time period, depended on the oxidative fatty acid metabolism of diatoms at sea.

2) Since some questions as to the applicability of the colorimetric assay for determining LOX activity arose during the analysis of field phytoplankton samples, a second part of the thesis consisted of analysing cultures of various diatom species, as well as clones of the same species. Samples were analyzed for oxylipin production and for LOX activity with the colorimetric assay and an alternative method. This aided in the evaluation of LOX activity assays and provided interesting data for examining species- and clone-specific differences in oxidative fatty acid metabolism in diatoms.

3) A third part of the thesis concentrated on examining the effect of various factors on oxylipin metabolism in diatoms. One of the aspects examined was nutrient limitation by analyzing cultures of *S. marinoi* grown under P-limitation in continuous culture. The effect of induced metabolic changes in diatoms on copepod reproduction was also examined by carrying out incubation experiments with *C. helgolandicus*. Another aspect considered was modulated oxylipin metabolism during different growth phases, both in the laboratory and in the field. Laboratory studies were carried out with *Pseudo-nitzschia delicatissima*, whereas field data were collected during a mesocosm experiment carried out at the University of Bergen as part of a EUROCEANS project on inducing a mono-algal bloom of *S. marinoi*.

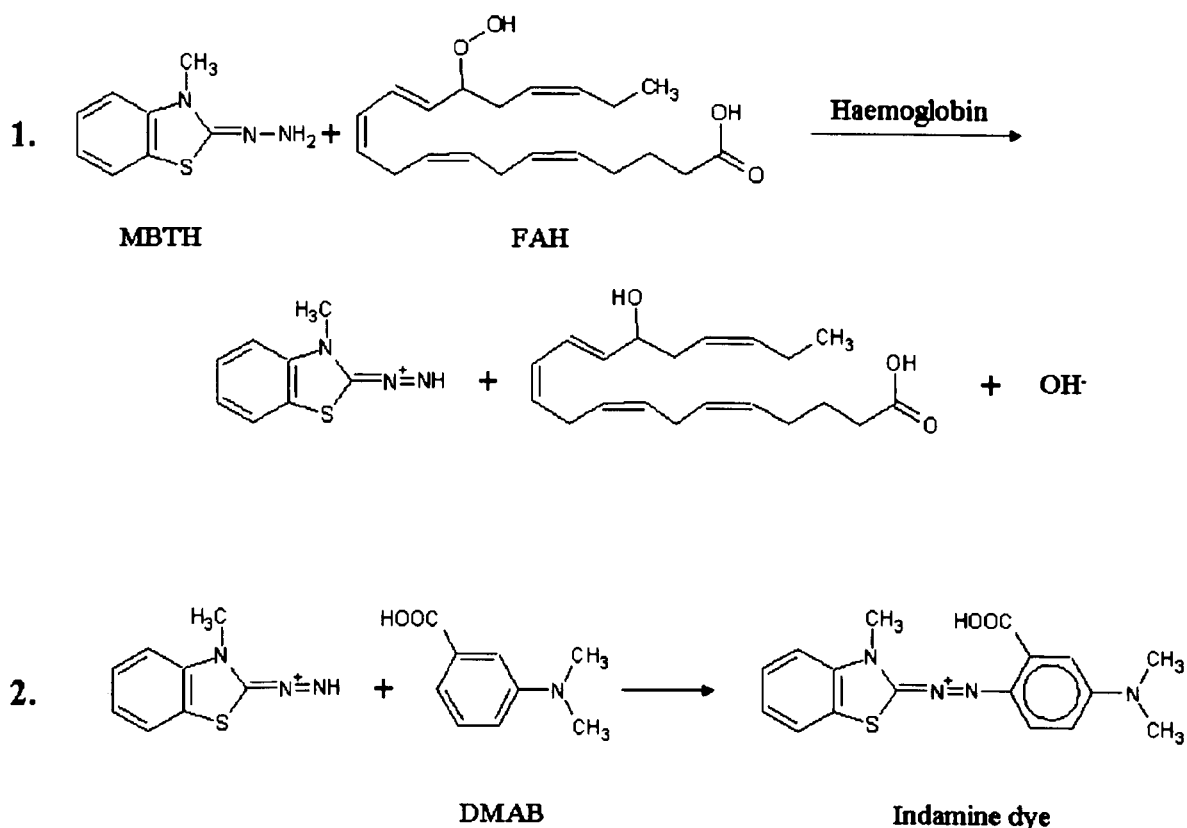
## **2 Lipoygenase Metabolism of *Skeletonema marinoi* during the Diatom Bloom in the Northern Adriatic Sea in 2005 and its Effects on the Reproductive Success of *Calanus helgolandicus***

In the Northern Adriatic Sea, yearly late winter diatom blooms have been found to negatively impact the reproductive success of *Calanus helgolandicus*. During the bloom in 2005, the lipoygenase (LOX) metabolism of the phytoplankton assemblage, dominated by *Skeletonema marinoi*, was therefore examined by the colorimetric assay and by spectrometric analyses of phytoplankton extracts. Copepod hatching success was strongly impacted during the peak of the bloom in 2005 and faecal pellet analysis in 2009 demonstrated that copepods apparently feed on *S. marinoi* during bloom periods. Aldehyde production was low in the collected phytoplankton samples in 2005 and the production of other oxylipins was detectable mainly at the beginning of the bloom. However, the LOX-derived intermediates of oxylipin metabolism, the fatty acid hydroperoxides (FAHs), detected colorimetrically, were correlated to the presence of diatoms at sea and reached their peak during the height of the bloom. Therefore, FAH production was also correlated to the decrease in hatching success, which was in phase with the presence of diatoms at sea. This decrease in hatching success may therefore have been due to a direct effect of FAHs or to a delayed effect of secondary oxylipins produced at the beginning of the bloom. The deleterious effect of the diatom bloom on copepod reproduction in 2005 was intermediate compared to the previous two years, which corresponded to an intermediate oxylipin production by the phytoplankton assemblage in 2005.

## 2.1 Introduction

Strong diatom blooms that negatively impact the hatching success of important zooplankton grazers, calanoid copepods, have been described as occurring in late winter in the Northern Adriatic Sea (Miralto *et al.* 1999). The deleterious effect of diatoms on copepod reproduction has been ascribed to the production of toxic secondary metabolites such as polyunsaturated aldehydes (PUAs) by this class of microalgae, which compromise hatching success of copepod eggs by inducing an apoptotic effect (Miralto *et al.* 1999).

Considering that the usual analysis of oxylipin production by mass spectrometry is quite elaborate, a colorimetric assay, based on the detection of lipoxygenase (LOX) activity was examined for its applicability to quantifying oxylipin metabolism in the phytoplankton assemblage. The colorimetric assay is both rapid and simple and requires only a spectrophotometer such as those commonly used in oceanic cruises. It may therefore be a valuable method for measuring the potential impact of diatoms on copepods directly at sea during field sampling. The colorimetric assay has been adapted from an assay proposed by Anthon and Barrett (2001) and is based on the indirect colorimetric detection of fatty acid hydroperoxides (FAHs), the products of LOX activity. These couple 3-methyl-2-benzothiazolinone (MBTH) and 3-(dimethylamino)-benzoic acid (DMAB) to a coloured compound in an oxidative reaction catalyzed by haemoglobin (Figure 2-1).



**Figure 2-1: Oxidative coupling due to fatty acid hydroperoxides (FAHs) of 3-methyl-2-benzothiazolinone (MBTH) and 3-(dimethylamino)-benzoic acid (DMAB) catalyzed by haemoglobin.**

The formation of the coloured compound can be detected spectrophotometrically. As colour formation is proportional to product formation by LOX enzymes, this assay provides a specific and sensitive method for determining LOX activity in phytoplankton samples. The coloured compound absorbs at 598 nm, thereby providing an advantage over the commonly used direct spectrophotometric detection of FAHs at 234 nm (Axelrod *et al.* 1981). Direct detection of FAHs at UV-range is not feasible in crude cell lysates because of interfering UV-absorbing material, which should not pose a problem at 598 nm (Anthon and Barrett 2001). Therefore the colorimetric assay should be applicable to the analysis of FAH production in phytoplankton samples.



To examine whether the colorimetric assay could provide a valid alternative to mass spectrometric oxylipin analysis, phytoplankton samples collected during the diatom bloom in the Northern Adriatic Sea in 2005 were analysed for LOX activity by the colorimetric assay and for oxylipin production by mass spectrometry. These results were compared with the hatching success of copepods measured at the time. Grazing by copepod females was not determined numerically. However, in 2009 faecal pellets were examined under the scanning electron microscope (SEM) to indirectly establish whether diatoms had been grazed. These results were then generalized for the yearly Adriatic diatom bloom, as least when the bloom was dominated by *Skeletonema marinoi* as was the case in both 2005 and 2009.

## 2.2 Materials and Methods

### 2.2.1 General

When not otherwise specified, chemicals were obtained from Sigma-Aldrich. Solvents were obtained from Carlo Erba reagents (Milan, Italy) or J. T. Baker (Deventer, Netherlands) and were GC-grade. A cooled centrifuge (DR 15P, Braun Biotechnology International) was used to separate the organic from the aqueous phase during extraction with organic solvents. Cell residue from sample incubations in Eppendorf tubes was removed in an ultracentrifuge (biofuge *fresco*, Heraeus). A spectrophotometer (Hewlett Packard 8453) was used to read absorbance at different wavelengths. Statistical analyses were carried out with GraphPad Prism 4.00 (GraphPad Software, Inc.).

Collection of field phytoplankton samples and incubation experiments for determining copepod reproduction were carried out by Antonio Miralto (SZN, Napoli). Phytoplankton

composition and cell concentrations in field phytoplankton samples were determined by Mauro Bastianini (ISMAR-CNR, Venezia). For the year 2005, no phytoplankton cell counts were available for the water column. However, cell counts of the collected phytoplankton net samples should be indicative of cell concentrations because samples were always collected in the same way (see 2.2.2.1). Values for cells ml<sup>-1</sup> in the following figures therefore do not refer to cell concentrations at sea, but to the concentrations in the phytoplankton net samples before centrifugation.

## **2.2.2 Field Phytoplankton Samples**

### **2.2.2.1 *Sample Collection***

Phytoplankton samples were collected at six stations along a transect from the mouth of the Po river to the centre of the Northern Adriatic Sea, whereby station 1 was the station farthest from shore and station 6 nearest to the coast (Table 2-1). Samples were collected by hauling a 20-µm phytoplankton net at the surface for 20 min. Net phytoplankton samples were concentrated in a 50-ml Falcon tube (BectonDickinson) by centrifugation and the pellets frozen in liquid nitrogen and stored at -80°C until analysis.

**Table 2-1: Latitude and longitude of six stations sampled in the Northern Adriatic Sea in 2005.**

Station	Latitude	Longitude
St. 1	44° 55',694	12° 56',672
St. 2	44° 55',824	12° 53',104
St. 3	44° 55',885	12° 49',430
St. 4	44° 55',990	12° 45',734
St. 5	44° 56',010	12° 42',032
St. 6	44° 56',278	12° 37',418

2.2.2.2 *Sample Preparation*

Frozen phytoplankton pellets were left to thaw at room temperature for 15 min before suspending cells in H<sub>2</sub>O<sub>deionised</sub> (Milli-Q) at one millilitre per gram sample (d'Ippolito *et al.* 2003). Suspended cells were sonicated for 1 min (2\*30s) at max. 20% output (Branson sonifier 250) on ice, carefully avoiding the formation of foam. Because sample volume increased after sonication, the total volume was measured to calculate the necessary aliquots in wet weight for the colorimetric assay (see 2.2.3). These were removed from the cell lysate and the colorimetric assay was started as soon as possible after sonication. Subsamples from the cell lysate were also taken for protein determination (30-50 µl) (see 2.2.6), which were frozen at -20°C in 1% protease inhibitor (Complete). Furthermore, subsamples were removed for chlorophyll *a* determination, which was carried out immediately (see 2.2.5). 30 min after sonication, acetone (1:1 v:v) was added to the remaining cell lysate, as well as decenal and 16-hydroxy-hexadecanoic acid (30-60 µg depending on total sample amount) as internal standards for the analysis of aldehydes and other oxylipins, respectively.

### 2.2.3 Colorimetric Lipoxygenase Activity Assay

The colorimetric assay had already been modified from Anthon and Barrett (2001) and adapted for the analysis of phytoplankton lysate (Fontana *et al.* 2007b) by Giuliana d'Ippolito at ICB-CNR, Pozzuoli (a previous OPEN University PhD student of Adrianna Ianora), who also carried out the calibration with eicosapentaenoic acid hydroperoxide, used for calculation of fatty acid hydroperoxide (FAH) concentrations in the samples (Figure 3-4).

Stock solutions were prepared as follows:

Stock A (20 mM DMAB in 100 mM phosphate buffer) was prepared by dissolving 330 mg DMAB (3-(dimethylamino)-benzoic acid) in 5 ml 1 N HCl, before diluting to 80 ml with H<sub>2</sub>O<sub>deionised</sub> and addition of 1.42 g Na<sub>2</sub>HPO<sub>4</sub>. The pH was adjusted to 6.0 with 1 N HCl before bringing the final volume to 100 ml with H<sub>2</sub>O<sub>deionised</sub>.

Stock B (10 mM MBTH) was prepared by dissolving 215.7 mg MBTH (3-methyl-2-benzothiazolinone) in 100 ml H<sub>2</sub>O<sub>deionised</sub>.

Haemoglobin (from bovine blood) stock solution was prepared at a concentration of 5 mg ml<sup>-1</sup> in H<sub>2</sub>O<sub>deionised</sub>.

EDTA stock solution was prepared at a concentration of 10 mM and sodium dodecyl sulphate (SDS) at a concentration of 1%, both in H<sub>2</sub>O<sub>deionised</sub>.

Eicosapentaenoic acid (25 mM EPA) was prepared according to Axelrod *et al.* (1981). EPA was suspended in Tween 20 (as 6% aqueous solution) at a 2:1 w:w ratio. The solution was clarified with 1 N NaOH (equimolar ratio) and brought to 25 mM final concentration with H<sub>2</sub>O<sub>deionised</sub>. The solution was divided into aliquots which were flushed with nitrogen and stored at -20°C.

Working solutions were prepared from stock solutions at the following concentrations:

Solution A: 10 mM DMAB in 50 mM sodium phosphate buffer by diluting stock A 1:1 with H<sub>2</sub>O<sub>deionised</sub>.

Solution B: 0.2 mM MBTH with 12.5 µg ml<sup>-1</sup> Haemoglobin by diluting stock B 1:50 in H<sub>2</sub>O<sub>deionised</sub> to which Haemoglobin stock solution was added at a 400-fold dilution.

For every sample, two concentrations were read corresponding to 4 and 8 mg sample wet weight (6-16 µl cell lysate, depending on sample concentrations) (Table 2-2). These aliquots were added to 0.4 ml solution A (DMAB) containing 10 µl EDTA (0.1 mM EDTA final concentration) in Eppendorf tubes. 16.2 µl EPA (0.4 mM final concentration) were added where required (see Table 2-2) before adding 0.5 ml of solution B (MBTH). Samples were thoroughly mixed and incubated at room temperature for 20 min. 0.5 ml sodium dodecyl sulfate (SDS 1%) was added to stop the reaction. Samples were centrifuged (10,000×g, 5 min, 4°C), supernatants were transferred to fresh Eppendorf tubes and absorbance was read at 598 nm. Blanks were read by adding cell lysate directly to the colorimetric reagents containing 1% SDS, centrifuging and reading absorbance immediately. All data points were run in duplicate.

**Table 2-2: Data points for the colorimetric assay. Blanks (4 mg blank and 8 mg blank) were read immediately, whereas all other samples were read after 20 min. Before reading the samples, 0.5 ml SDS 1% were added, which were already contained in the reagent mix for blanks.**

Sample	Sol A [μl]	EDTA [μl]	1% SDS [μl]	EPA [μl]	Cells [mg]	Sol B [μl]
Solution blank	400	10				500
EPA blank	400	10		16.2		500
4 mg blank	400	10	500		4	500
8 mg blank	400	10	500		8	500
4 mg	400	10			4	500
4 mg+EPA	400	10		16.2	4	500
8 mg	400	10			8	500
8 mg+EPA	400	10		16.2	8	500

For comparative purposes of field phytoplankton samples, lipoxygenase (LOX) activity determined colorimetrically was defined as follows. LOX activity was calculated from the absorbance read in the sample at 4 mg wet weight after 20 min (Table 2-2: 4 mg) without considering the blanks (for an explained reasoning see Chapter 3). Absorbance read in these samples after 20 min was corrected only for the absorbance due to the solution blank. FAH concentration in the sample was calculated from absorbance at 598 nm by means of a calibration curve carried out with eicosapentaenoic acid hydroperoxide (Figure 3-4). Changes in FAH concentrations in the cell lysate on incubation with exogenous fatty acids (Table 2-2: 4mg+EPA) were not considered in the calculations for LOX activity in the phytoplankton field samples (see 3.4). FAH production was normalized for protein or chlorophyll *a*.

## 2.2.4 Oxylipin Analysis

### 2.2.4.1 Sample Extraction

The cell lysate, to which acetone (1:1 v:v) had been added (see 2.2.2.2), was centrifuged (2750×g, 6 min, 4°C) and the pellet extracted two more times with H<sub>2</sub>O<sub>deionised</sub>/acetone (1:1 v:v), suspending cells first in H<sub>2</sub>O<sub>deionised</sub> before adding acetone and mixing vigorously before centrifuging. The supernatants were pooled and extracted three times with CH<sub>2</sub>Cl<sub>2</sub> (1:1 v:v). After each centrifugation step to separate phases (same settings as above), the upper water phase was transferred with a Pasteur pipette into a fresh Falcon tube before extracting again with CH<sub>2</sub>Cl<sub>2</sub>. The organic phases were combined, dried over Na<sub>2</sub>SO<sub>4</sub> and filtered. The solvent was removed under reduced pressure (Büchi Rotavapor R-114) until dryness. Extracts were dissolved in CH<sub>2</sub>Cl<sub>2</sub>, divided 1:2 into two pre-weighed glass vials, the solvent removed under reduced pressure and the weight of the vials determined. The smaller sample amount (one-third) was flushed with nitrogen and frozen at -80°C until derivatisation with ethereal diazomethane for analysis of oxylipins other than aldehydes on LC-MS/MS. The larger sample amount (two-thirds) was derivatised immediately with (1-ethoxycarbonylethyliden)-triphenyl-phosphorane (CET) (1.1:1 w:w) in CH<sub>2</sub>Cl<sub>2</sub> for 20 hours at room temperature according to d'Ippolito *et al.* (2002a) for the analysis of volatile aldehydes. After removal of the solvent, the sample was flushed with nitrogen and frozen at -80°C until GC-MS analysis.

### 2.2.4.2 Quantification of Aldehydes and Other Oxylipins

All analyses of metabolites were conducted at the ICB-CNR (Pozzuoli) under the direction of Angelo Fontana according to protocols established by his group. Oxylipin molecules

had been previously characterized (d'Ippolito *et al.* 2002a; d'Ippolito *et al.* 2002b; d'Ippolito *et al.* 2005). Oxylipins other than aldehydes quantified in samples were the hydroxy-derivatives and hydroxy-epoxy-derivatives (epoxyalcohols) of hexadecatetraenoic acid (HTA), hexadecatrienoic acid (HTrA), and eicosapentaenoic acid (EPA) (Table 2-3). These were detected as methylated derivatives and identified with the help of retention times and mass spectra on a Qtof-*micro* mass spectrometer (Waters SpA, Milan, Italy), equipped with an ESI source (positive mode) and coupled to a Waters Alliance HPLC system. The internal standard (hydroxy-C<sub>16:0</sub>) showed a molecular ion mass at 309 m/z.

**Table 2-3: Molecular ion mass [m/z] detected in LC-MS analysis of extracts and used for quantification of the hydroxy-acids and epoxyalcohols derived from hexadecatetraenoic acid (HTA), hexadecatrienoic acid (HTrA), and eicosapentaenoic acid (EPA). Abbreviations in brackets indicate oxylipin species identified on the chromatograms in the Appendix.**

Fatty acid	Hydroxy-acid	Epoxyalcohol
HTA (C <sub>16:4</sub> )	301 (HTA-OH)	317 (HTA-EPOX)
HTrA (C <sub>16:3</sub> )	303 (HTrA-OH)	319 (HTrA-EPOX)
EPA (C <sub>20:5</sub> )	355 (EPA-OH)	371 (EPA-EPOX)

Aldehydes were detected as CET-derivatives on a GC-MS (Hewlett & Packard 5989B mass spectrometer with a 5890 Series II Plus gas chromatograph). Aldehyde species quantified in samples were heptadienal, octadienal, octatrienal, and decatrienal (Table 2-4).



**Table 2-4 Molecular ion mass [m/z] and retention times [min] detected in GC-MS analysis of extracts and used for quantification of the aldehydes octatrienal, octadienal, heptadienal, and decatrienal, derived from the respective fatty acids hexadecatetraenoic acid (HTA), hexadecatrienoic acid (HTrA), and eicosapentaenoic acid (EPA). Abbreviations in brackets indicate aldehyde species identified on the chromatograms in the Appendix.**

Fatty acid	Aldehyde	Molecular ion mass [m/z]	Retention Time [min]
HTA (C <sub>16:4</sub> )	Octatrienal (OT)	206	~5-6
HTrA (C <sub>16:3</sub> )	Octadienal (OD)	208	~5-6
EPA (C <sub>20:5</sub> )	Heptadienal (HD)	194	~4-5
	Decatrienal (DT)	234	~8

The internal standard (decenal) showed a molecular ion mass at 238 m/z at ~6 min. Quantification was carried out by comparison of integrated peak areas of oxylipins with internal standards. Oxylipin production in the phytoplankton samples was normalized either for protein or for diatom cells.

### 2.2.5 Chlorophyll *a* Determination

Varying concentrations of cell lysate were extracted in NaHCO<sub>3</sub>-saturated 80% acetone for 2 h at 4°C in the dark in Eppendorf tubes. After centrifugation (10,000×g, 20 min, 4°C), absorbance of supernatants was read at two wavelengths (630 and 664 nm) and chlorophyll *a* concentration in the cell lysate was calculated as:

$$\text{chl } a \text{ (}\mu\text{g l}^{-1}\text{)} = ( V_{\text{aliquot}} [\text{l}] / V_{\text{read}} [\mu\text{l}] ) * ( 11.47 \lambda_{664} - 0.4 \lambda_{630} )$$

with  $\lambda_{664}$  and  $\lambda_{630}$  the absorbance at 664 nm (chl *a*) and 630 nm (chl *c*),  $V_{\text{aliquot}}$  the amount of sample and  $V_{\text{read}}$  the total sample volume (Jeffrey and Humphrey 1975).

### **2.2.6 Protein Determination**

Protein content in phytoplankton samples was determined according to the Bradford method (Bio-Rad) with bovine serum albumin (BSA) as standard. Samples of cell lysate were treated with 5% Triton for 20 min prior to analysis, diluted 1:5 with  $\text{H}_2\text{O}_{\text{deionised}}$  and centrifuged ( $10,000\times g$ , 5 min,  $4^\circ\text{C}$ ). Supernatants were incubated at 3-4 different concentrations with the colorimetric reagents according to manufacturer's instructions in Eppendorf tubes and absorbance was read at 655 nm. Standard curves with  $1\text{ mg ml}^{-1}$  BSA were read with each analysis and used to calculate protein concentrations in the cell lysate.

### **2.2.7 Copepod Reproduction**

For each station and sampling date, females of *Calanus helgolandicus* were incubated individually in Falcon tissue culture flasks in 50 ml seawater containing natural phytoplankton assemblage. Eggs were counted after 24 h, whereas the percentage of hatched eggs was determined after 48 h.

### **2.2.8 Copepod Grazing Determined by SEM**

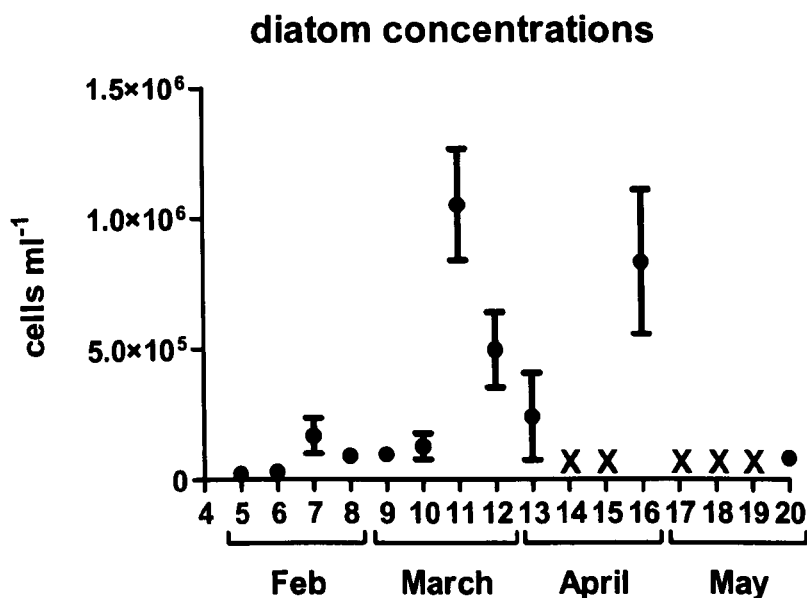
Faecal pellets were collected from females used for determining copepod reproductive success during the diatom bloom in the Northern Adriatic Sea in 2009. Samples had been left 3 days at room temperature (see 2.2.7), which was more than sufficient for bacterial

degradation of peritrophic membranes which can otherwise interfere with analyses (Turner and Ferrante 1979). Faecal pellets were sorted under the microscope into filtered seawater and stored in 4% formalin (final concentration) for two weeks at 4°C. Faecal pellets were isolated from four different dates and stations during the diatom bloom to observe differences among stations as well as changes during the course of the bloom. Samples were prepared for SEM analysis by filtering them onto 3.0-µm isopore<sup>TM</sup> membrane filters (Millipore), which were treated by ethanol (EtOH) dehydration series (25, 50, 95, and 100%) and kept in 100% EtOH until mounting, critical point drying and sputtering with gold (Turner 1978). *Skeletonema marinoi* was identified in the samples with the help of Diana Sarno (SZN, Napoli) by examining the ultrastructure of the diatom frustule.

## 2.3 Results

### 2.3.1 Diatom Bloom Development

During the observed time period (February-May 2005), phytoplankton was composed almost exclusively of diatoms with few other phytoplankton species present, such as the dinoflagellates *Alexandrium spp.* and *Prorocentrum micans*.



**Figure 2-2: Mean diatom cell concentrations ( $\pm$ SEM,  $n=6$ ) of stations 1-6 [cells  $\text{ml}^{-1}$ ] in the phytoplankton net samples in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).**

Diatom cell concentrations in the net samples started to increase towards mid-March whereupon the major peak was reached ( $1.1 \pm 0.2 \times 10^6$  cells  $\text{ml}^{-1}$ ) (Figure 2-2). Cell concentrations then decreased before reaching a second, minor peak at the end of April ( $0.8 \pm 0.3 \times 10^6$  cells  $\text{ml}^{-1}$ ). At the end of May, diatom concentrations had returned to pre-bloom values ( $0.8 \pm 0.3 \times 10^5$  cells  $\text{ml}^{-1}$ ). Diatom cell numbers were an order of magnitude higher during the bloom compared to non-bloom values.

### 2.3.2 Phytoplankton Composition

There were no statistically significant differences between diatom cell concentrations in net samples from different stations (ANOVA:  $p > 0.05$ ). However, as actual cell counts at sea were not available, the phytoplankton composition during the development of the

bloom was examined at the different stations (Figure 2-3). Once again, concentrations in net samples were considered representative of concentrations at sea. Phytoplankton groups identified were *Skeletonema marinoi*, *Chaetoceros spp.*, *Pseudo-nitzschia spp.*, *Thalassiosira rotula*, and other minor phytoplankton groups which included non-diatoms.

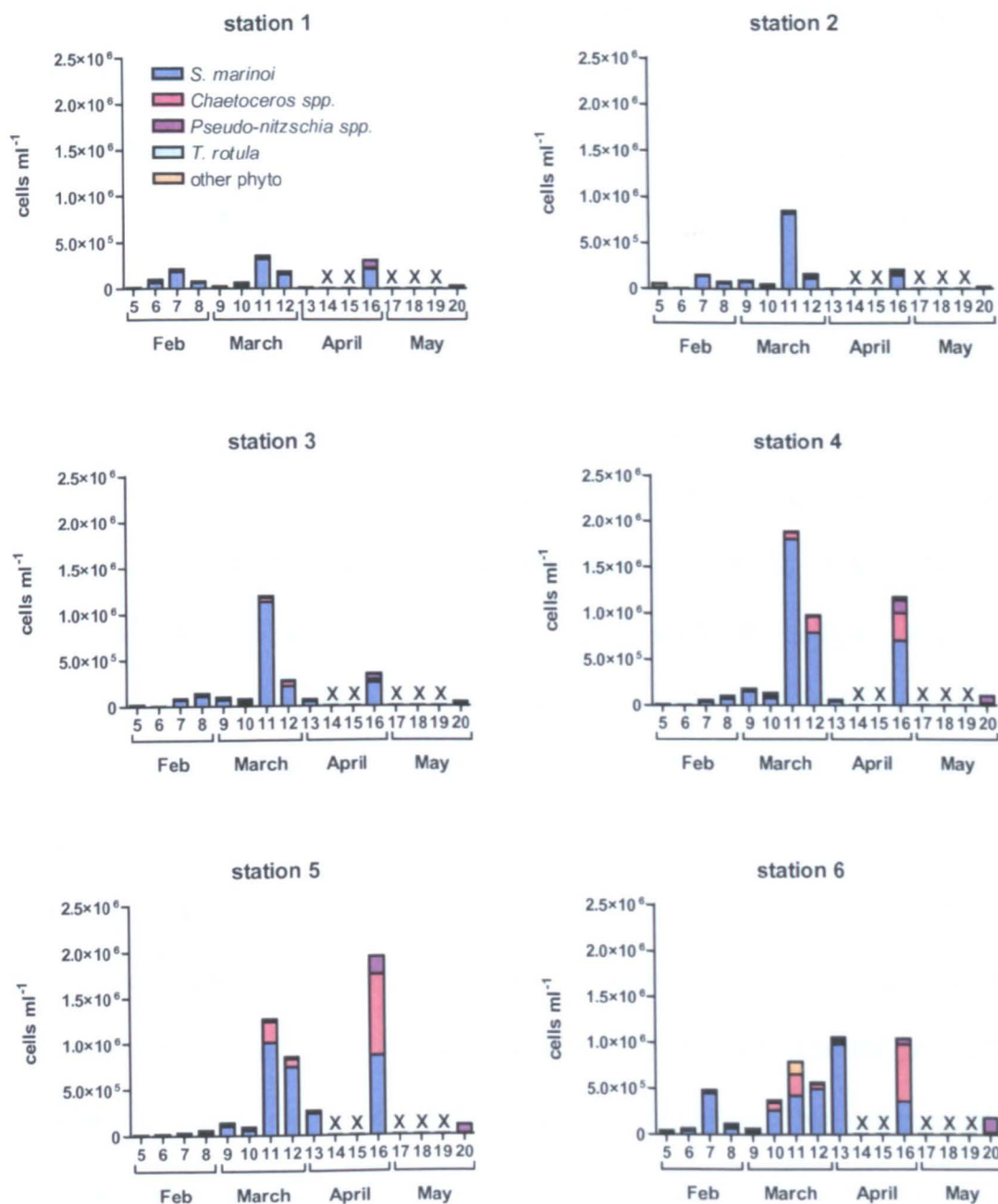


Figure 2-3: Phytoplankton composition at stations 1-6 in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19). Species identified included *Skeletonema marinoi*, *Chaetoceros* spp., *Pseudo-nitzschia* spp., *Thalassiosira rotula*, and other minor phytoplankton species including non-diatoms.

At station 1, *S. marinoi* was the dominant phytoplankton species present in net samples during the entire time period with *Pseudo-nitzschia spp.* appearing in minor amounts (~25%) towards the end of the bloom in late April. Peak total concentrations in net samples were reached in mid-March and at the end of April. In general, phytoplankton concentrations in net samples at this station were the lowest of all stations examined (peak concentrations:  $3.6 \times 10^5$  cells  $\text{ml}^{-1}$ ). At station 2, peak phytoplankton concentrations in net samples were found in mid-March ( $8.5 \times 10^5$  cells  $\text{ml}^{-1}$ ), consisting almost entirely of *S. marinoi*. Station 3 had highest phytoplankton concentrations in collected net samples in mid-March as well ( $1.2 \times 10^6$  cells  $\text{ml}^{-1}$ ), consisting almost entirely of *S. marinoi* with a minimal contribution of *Chaetoceros spp.* At station 4, highest phytoplankton concentrations in net samples were also found in mid-March ( $1.9 \times 10^6$  cells  $\text{ml}^{-1}$ ) with another minor peak at the end of April ( $1.2 \times 10^6$  cells  $\text{ml}^{-1}$ ). The second peak was still dominated by *S. marinoi*, but with a significant contribution of *Chaetoceros spp.* (~25%) and a minor contribution of *Pseudo-nitzschia spp.* (~10%). Station 5 differed from the other stations in that highest phytoplankton concentrations in net samples were found at the end of April ( $1.9 \times 10^6$  cells  $\text{ml}^{-1}$ ) with a minor peak in mid-March. Also the phytoplankton composition differed in that it was less dominated by *S. marinoi*. This was especially true of the later peak which was composed mainly of *Chaetoceros spp.* (~50%) with a minor contribution of *Pseudo-nitzschia spp.* (~10%). *S. marinoi* made up only ~40% of total phytoplankton during this peak. Also at station 6, the contribution of *Chaetoceros spp.* to the phytoplankton bloom at the end of April was high. Whereas peak phytoplankton concentrations ( $1.0 \times 10^6$  cells  $\text{ml}^{-1}$ ) at the beginning of April were due almost entirely to *S. marinoi*, phytoplankton composition at the end of the month had shifted to ~60% *Chaetoceros spp.*

### 2.3.3 Oxylipin Production

#### 2.3.3.1 Aldehyde Production

Aldehyde production in general was low in the samples. The type of aldehydes detected were heptadienal, octadienal, octatrienal, and in the early samples also decatrienal. In Appendix 1, a representative chromatogram is presented of a station at which phytoplankton lysate was producing aldehydes (station 6: 11.02.05, week 6) and a station at which no aldehyde production was observed (station 1: 20.05.05, week 20). Only in ~30% of the samples were detectable amounts of aldehydes recorded, mainly at station 6 during February and the beginning of March (Figure 2-4). This coincided with the highest *Skeletonema* concentrations ( $4.5 \times 10^5$  cells ml<sup>-1</sup>, Figure 2-3) recorded in net samples at this station compared to other stations in February. However, although aldehyde production was expected to increase with increasing biomass of *S. marinoi* at this station, as well as at the other stations, this was not the case. From the end of March onward, aldehydes were almost absent from the phytoplankton samples (Figure 2-4).



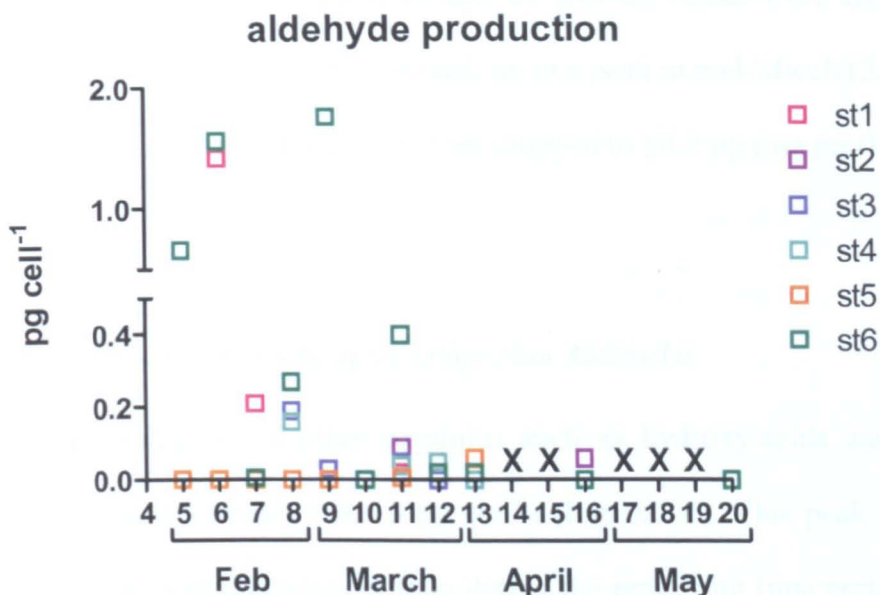


Figure 2-4: Aldehyde production ( $n=1$ ) normalized for diatom cells [ $\text{pg cell}^{-1}$ ] in phytoplankton samples collected at stations 1-6 in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).

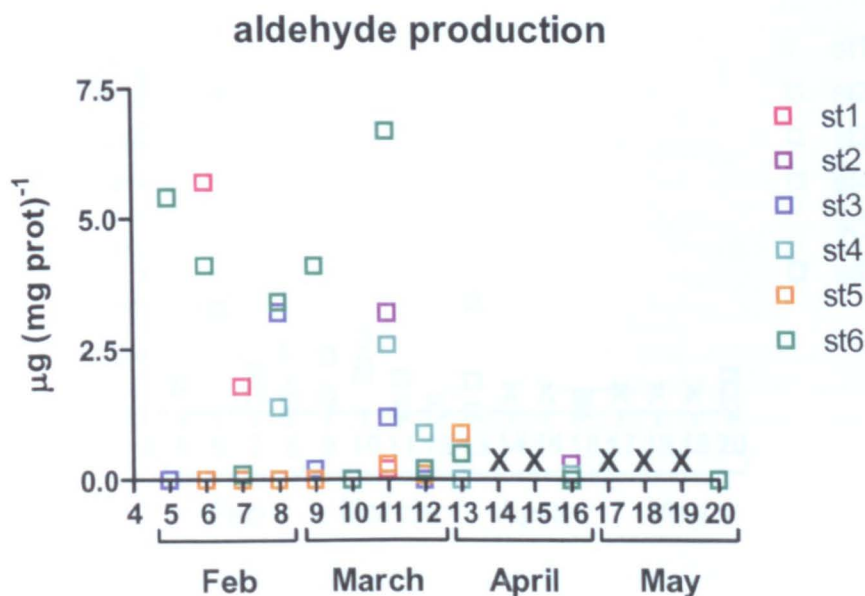


Figure 2-5: Aldehyde production ( $n=1$ ) normalized for protein [ $\mu\text{g (mg prot)}^{-1}$ ] in phytoplankton samples collected at stations 1-6 in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).

Also when aldehydes were normalized for protein, values were apparently higher at the beginning of the time period examined, up to a peak in mid-March ( $2.4 \pm 1.0 \mu\text{g (mg prot)}^{-1}$ ) (Figure 2-5). Aldehyde production then dropped to  $\leq 0.2 \mu\text{g (mg prot)}^{-1}$  in April and May.

2.3.3.2 Production of Oxylipins Other than Aldehydes

Cellular production of other oxylipins such as hydroxy-acids and epoxyalcohols also peaked in mid-February ( $28 \pm 13 \text{ pg cell}^{-1}$ ) (Figure 2-6). This peak was very pronounced with values thirty times higher than during the remaining time period ( $1.0 \pm 0.6 \text{ pg cell}^{-1}$ ). After the peak, values were more or less constant.

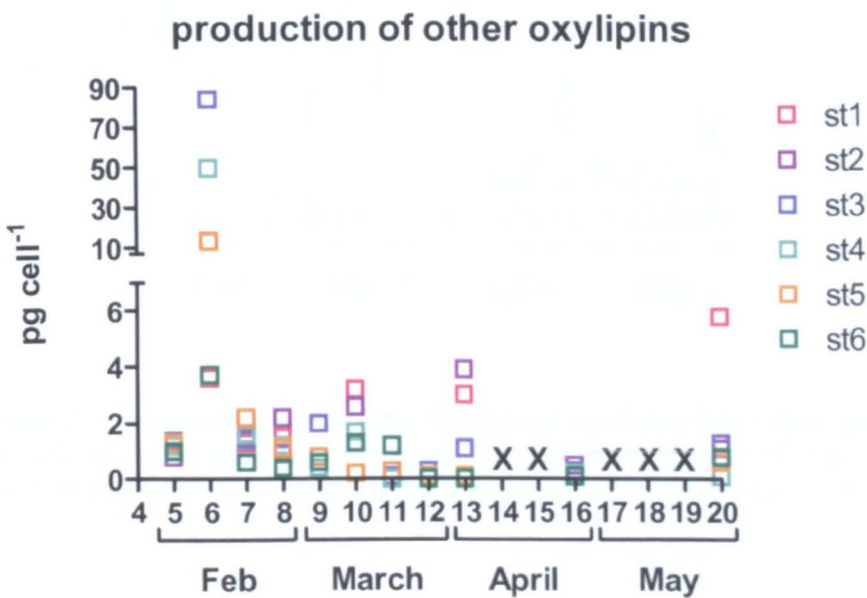
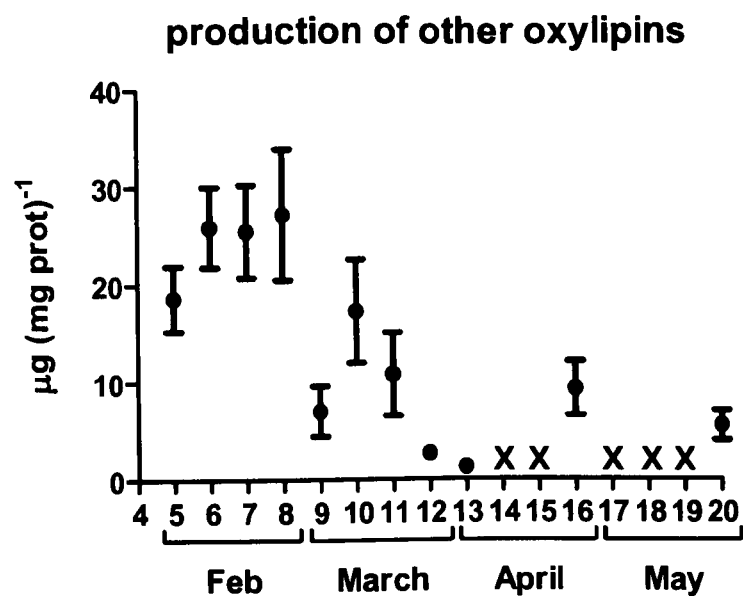


Figure 2-6: Production of oxylipins other than aldehydes (n=1) normalized for diatom cells [ $\text{pg cell}^{-1}$ ] in phytoplankton samples collected at stations 1-6 in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).

High mean values in mid-February were mainly due to a high production of oxylipins at stations 3 and 4, although stations 2 and 5 also showed high oxylipin production of  $\sim 13 \text{ pg cell}^{-1}$  (Figure 2-6). However, there were no statistically significant differences between stations when the entire time period was considered (ANOVA:  $p > 0.05$ ) and at all stations, peak production was found in mid-February. Mean production of oxylipins other than aldehydes in 2005 was  $3.4 \pm 2.5 \text{ pg cell}^{-1}$ .



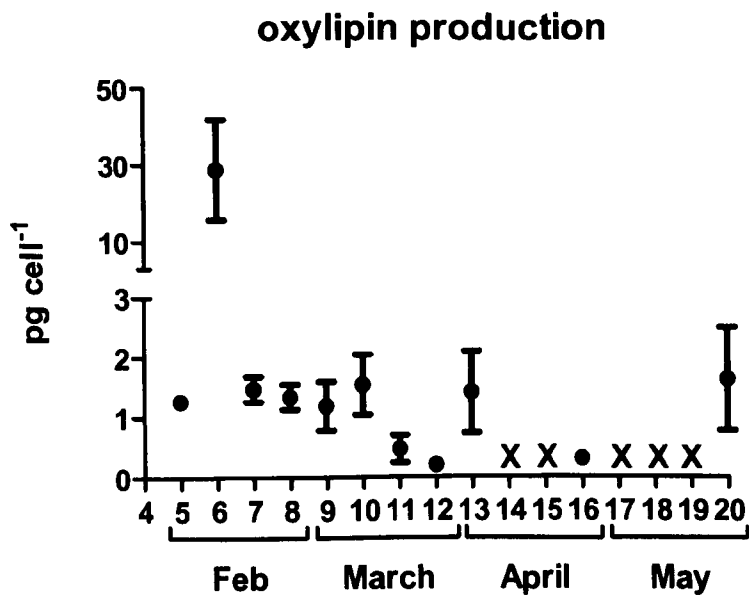
**Figure 2-7: Mean production ( $\pm$ SEM,  $n=6$ ) of oxylipins other than aldehydes of stations 1-6 normalized for protein [ $\mu\text{g (mg prot)}^{-1}$ ] in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).**

Mean production of other oxylipins normalized for protein also showed higher values in February with a peak of  $27 \pm 7 \text{ }\mu\text{g (mg prot)}^{-1}$  at the end of the month (Figure 2-7). Oxylipin production normalized for protein decreased during March and then remained low until the end of May. There were no statistically significant differences between stations (ANOVA:  $p > 0.05$ ). Mean production of oxylipins other than aldehydes in 2005 was  $13.2 \pm 3.0 \text{ }\mu\text{g}$

(mg prot)<sup>-1</sup>. Representative chromatograms for dates in which production of oxylipins other than aldehydes was pronounced (24.02.05: week 8) and low (1.04.05: week 13) are to be found in Appendix 1.

2.3.3.3 *Oxylipin Production*

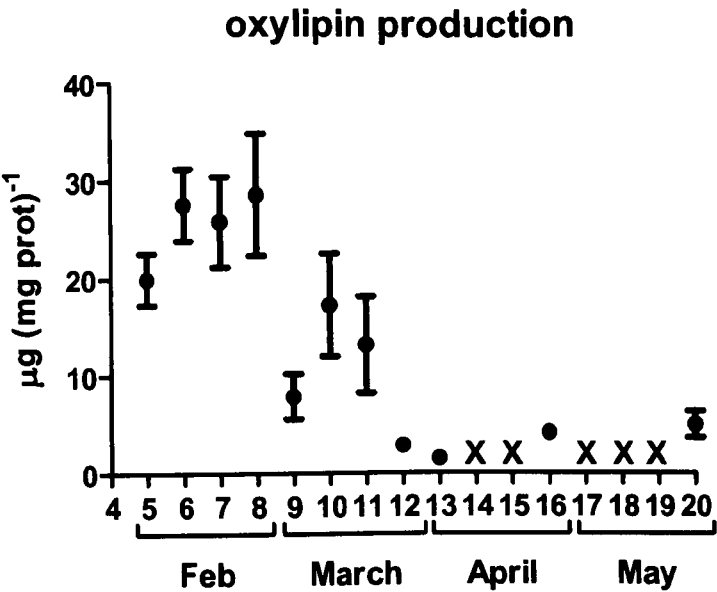
When aldehydes and other oxylipins were considered together, the pattern remained similar to that of just other oxylipins because of the low contribution of aldehydes to oxylipin production (Figures 2-8 and 2-9).



**Figure 2-8: Mean oxylipin production ( $\pm$ SEM, n=6) of stations 1-6 normalized for diatom cells [pg cell<sup>-1</sup>] in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).**

Oxylipin production was therefore mainly due to oxylipins other than aldehydes such as hydroxy-acids and epoxyalcohols. Mean cellular production only increased from  $3.4 \pm 2.5$

to  $3.6 \pm 2.5 \text{ pg cell}^{-1}$  when aldehydes were included in oxylipin production (Figure 2-8). Except for the high peak in mid-February ( $30 \pm 13 \text{ pg cell}^{-1}$ ), cellular production of oxylipins was relatively constant from the beginning of February to the end of May at  $1.1 \pm 0.2 \text{ pg cell}^{-1}$ .

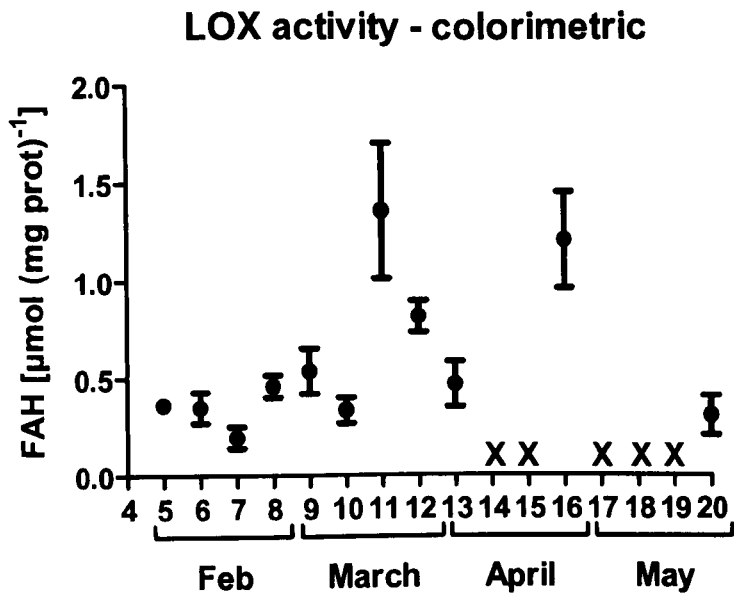


**Figure 2-9: Mean oxylipin production ( $\pm$ SEM,  $n=6$ ) of stations 1-6 normalized for protein [ $\mu\text{g (mg prot)}^{-1}$ ] in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19).**

Mean oxylipin production normalized for protein increased from  $13.2 \pm 3.0$  to  $14.0 \pm 3.1 \text{ }\mu\text{g (mg prot)}^{-1}$  when aldehyde production was included (Figure 2-9). The bulk of oxylipin production was observed in February peaking at the end of the month ( $28.6 \pm 6.2 \text{ }\mu\text{g (mg prot)}^{-1}$ ) with a decrease during March to reach low values in April and May ( $\leq 5 \text{ }\mu\text{g (mg prot)}^{-1}$ ).

2.3.4 Lipoxygenase Activity

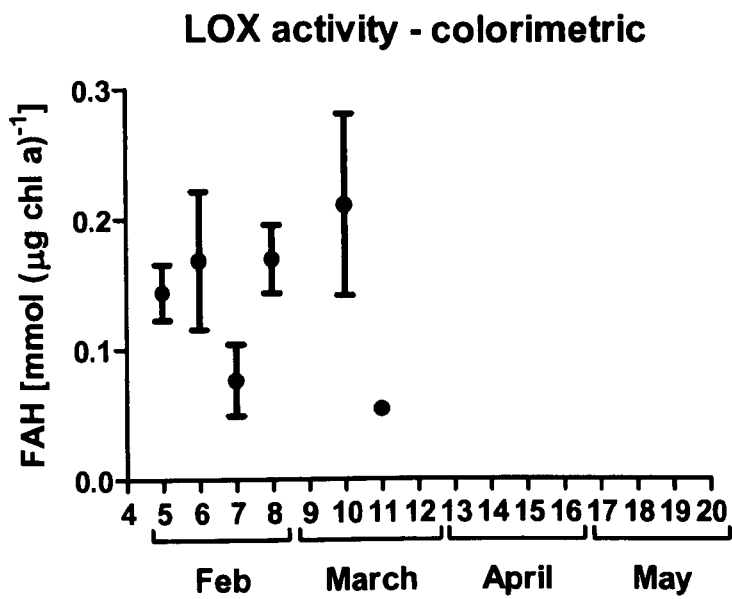
Lipoxygenase (LOX) activity determined with the colorimetric assay was not significantly different between stations (ANOVA:  $p>0.05$ ) and therefore data for all stations were pooled (Figure 2-10).



**Figure 2-10: Mean fatty acid hydroxide (FAH) production ( $\pm$ SEM,  $n=6$ ) of stations 1-6 normalized for protein [ $\mu\text{mol (mg prot)}^{-1}$ ] determined with the colorimetric assay in February-May 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15 and 17-19). FAH production was calculated from FAH concentrations in cell lysates after 20 min without considering blanks.**

The colorimetric assay demonstrated highest LOX activity towards the end of the observed time period with the highest peak in mid-March ( $1.4\pm0.3 \mu\text{mol FAH (mg prot)}^{-1}$ ) and a slightly lower peak at the end of April ( $1.2\pm0.2 \mu\text{mol FAH (mg prot)}^{-1}$ ) (Figure 2-10). Mean FAH production in 2005 was  $0.58\pm0.12 \mu\text{mol FAH (mg prot)}^{-1}$ .

Chlorophyll *a* concentrations were only analyzed in the early phytoplankton samples (February to mid-March). However, when FAH production was normalised for chlorophyll *a*, a similar pattern as in Figure 2-10 was observed for LOX activity in phytoplankton samples during this time period (Figure 2-11).



**Figure 2-11: Mean fatty acid hydroxide (FAH) production ( $\pm$ SEM,  $n=6$ ) of stations 1-6 normalized for chlorophyll *a* [ $\mu\text{mol (ng chl } a)^{-1}$ ] determined with the colorimetric assay in February-May 2005 (numbers on x-axis indicate weeks of the year; sample analysis was only carried out up to week 11). FAH production was calculated from FAH concentrations in cell lysates after 20 min without considering blanks.**

2.3.5 Copepod Reproduction

2.3.5.1 Egg Production Rates

Egg production rates did not differ significantly between stations (ANOVA:  $p>0.05$ )  
Therefore mean egg production rates of all stations were calculated (Figure 2-12).

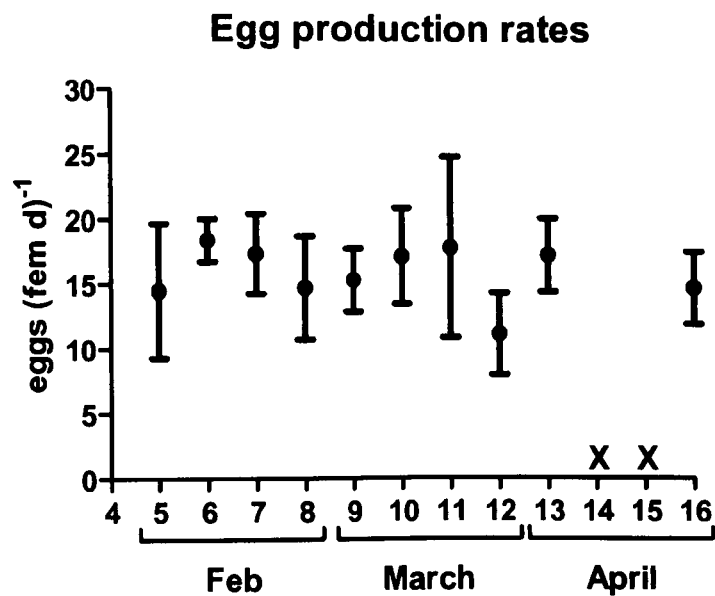


Figure 2-12: Mean egg production rates ( $\pm$ SEM,  $n=6$ ) of *Calanus helgolandicus* females [eggs (fem d)<sup>-1</sup>] collected at stations 1-6 in February-April 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15).

Mean egg production rates were constant at  $16\pm2$  eggs female<sup>-1</sup> day<sup>-1</sup> from the beginning of February to the end of April (Figure 2-12).



2.3.5.2 Egg Hatching Success

Also hatching success of copepod eggs did not differ significantly between stations (ANOVA:  $p>0.05$ ). Mean hatching success was highest at the end of February with  $78\pm4\%$  of eggs developing to hatching (Figure 2-13).

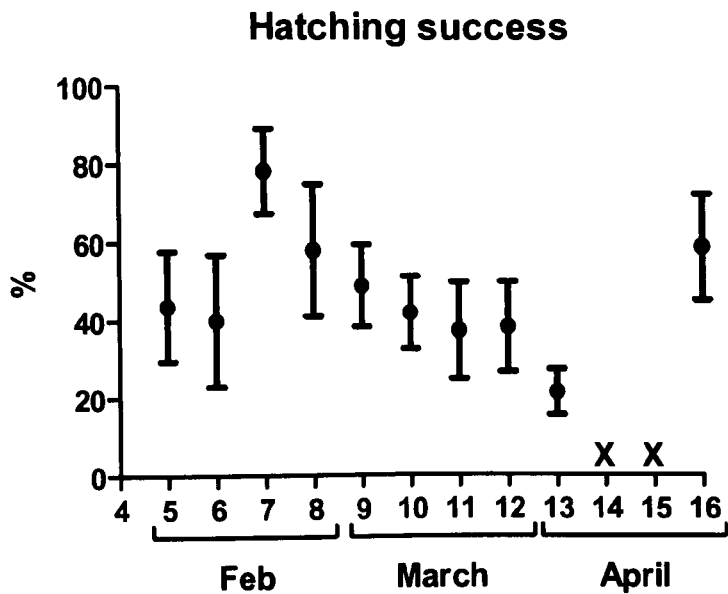
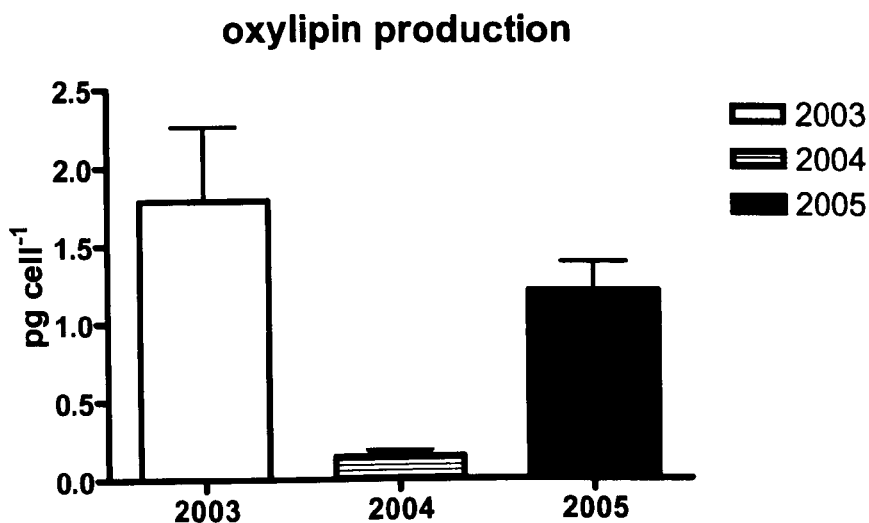


Figure 2-13: Mean hatching success ( $\pm$ SEM,  $n=6$ ) of copepod eggs produced by *Calanus helgolandicus* females [%] collected at stations 1-6 in February-April 2005 (numbers on x-axis indicate weeks of the year). Crosses represent absence of sampling (weeks 14-15).

Hatching success decreased to a low of  $21\pm2\%$  at the beginning of April before recovering to higher values ( $59\pm6\%$ ) at the end of the month. Mean hatching success over the entire time period in 2005 was  $47\pm5\%$ .

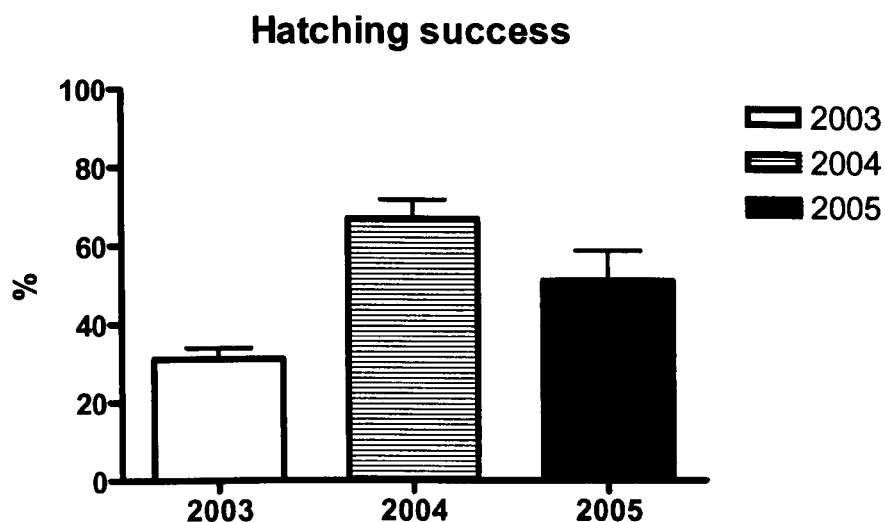
### 2.3.6 Comparison between Years

Oxylipin production of phytoplankton samples had also been determined during the diatom blooms in 2003 and 2004. Therefore oxylipin production and hatching success were compared between the three years. Statistical analyses were carried out considering only the weeks of the year in which data were available for both hatching success and oxylipin production in all three years examined (n=6).



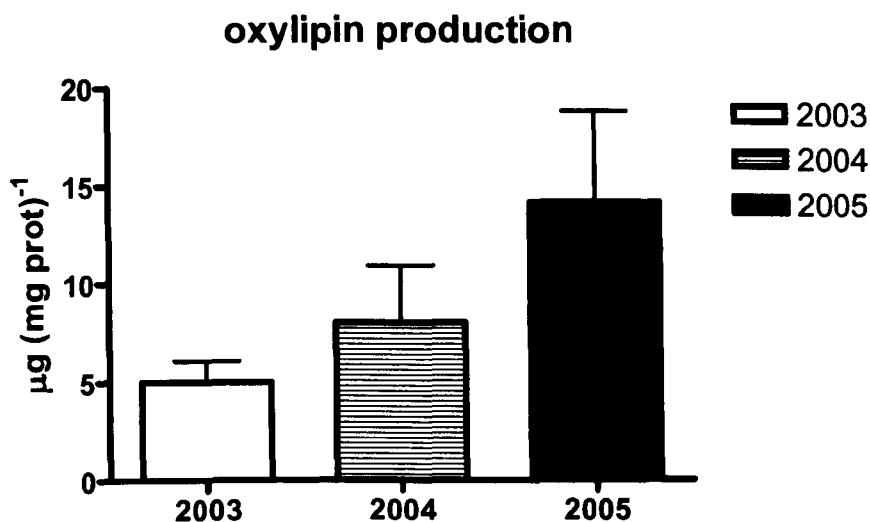
**Figure 2-14: Yearly mean oxylipin production (+SEM, n=6) normalized for diatom cells [pg cell<sup>-1</sup>] in phytoplankton samples collected at stations 1-6 during the late winter diatom blooms in 2003, 2004, and 2005.**

Yearly mean cellular production of oxylipins in 2003 was  $1.8 \pm 0.5$  pg cell<sup>-1</sup>, whereas cellular oxylipin production was significantly lower in 2004 at  $0.14 \pm 0.09$  pg cell<sup>-1</sup> (Figure 2-14) (repeated measures ANOVA:  $F=6.0$ ,  $r^2=0.60$ ,  $p=0.03$  and Tukey's post-test:  $p<0.05$ ). Oxylipin production in 2005 for the considered time period was  $1.2 \pm 0.5$  pg cell<sup>-1</sup> and thereby did not differ significantly from either of the previous two years.



**Figure 2-15: Yearly mean hatching success (+SEM, n=6) of copepod eggs produced by *Calanus helgolandicus* females [%] collected at stations 1-6 during the late winter diatom blooms in 2003, 2004, and 2005.**

Mean hatching success was low in 2003 at  $31 \pm 3\%$ , whereas in 2004 hatching success was significantly higher at  $67 \pm 5\%$  (repeated measures ANOVA:  $F=16.4$ ,  $r^2=0.80$ ,  $p=0.0015$  and Tukey's post-test:  $p<0.01$ ). Hatching success was intermediate in 2005 at  $51 \pm 8\%$  (Figure 2-15) and did not differ significantly from either of the two previous years.



**Figure 2-16: Yearly mean oxylipin production (+SEM, n=6) normalized for protein [ $\mu\text{g (mg prot)}^{-1}$ ] in phytoplankton samples collected at stations 1-6 during the late winter diatom blooms in 2003, 2004, and 2005.**

A different pattern was observed when oxylipins were normalized for protein (Figure 2-16) with no statistically significant differences between the years (repeated measures ANOVA:  $p > 0.05$ ).

### 2.3.7 Copepod Grazing

Composition of faecal pellets collected from females reflected the predominance of *Skeletonema marinoi* at sea. Since there seemed to be no differences in the composition of faecal pellets between stations, SEM photographs from one representative station (station 2) were compared for different sampling dates during the course of the bloom (Figures 2-17 to 2-20).

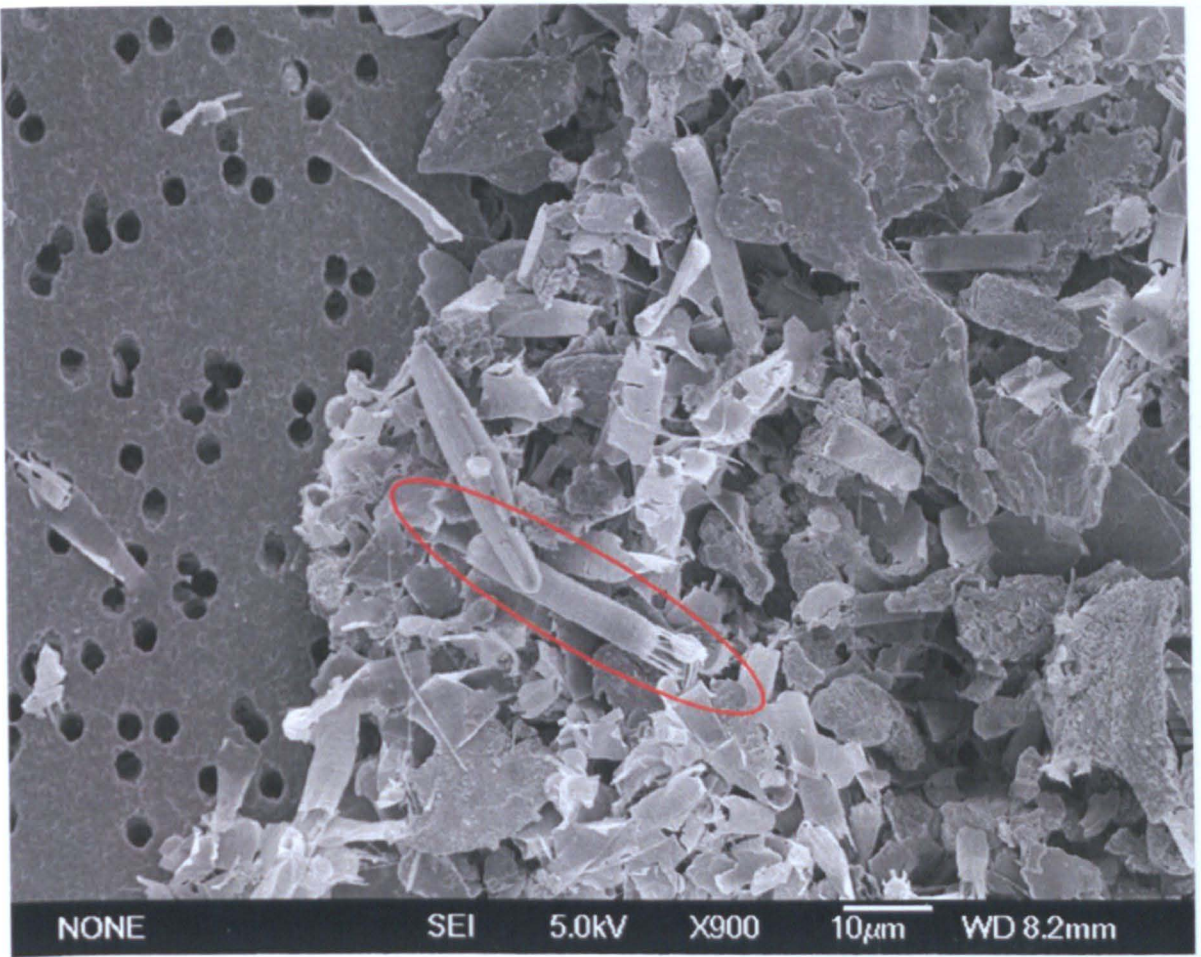
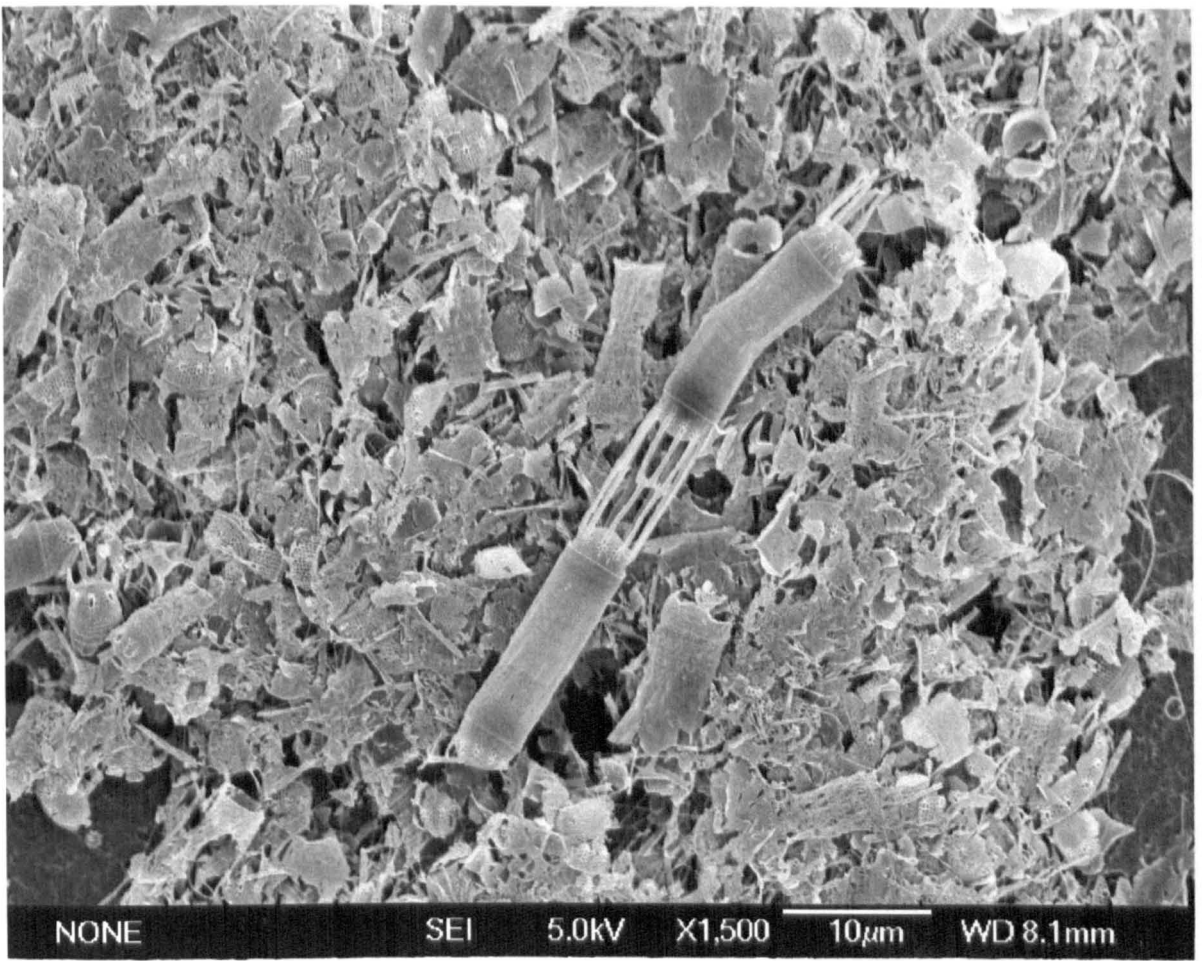


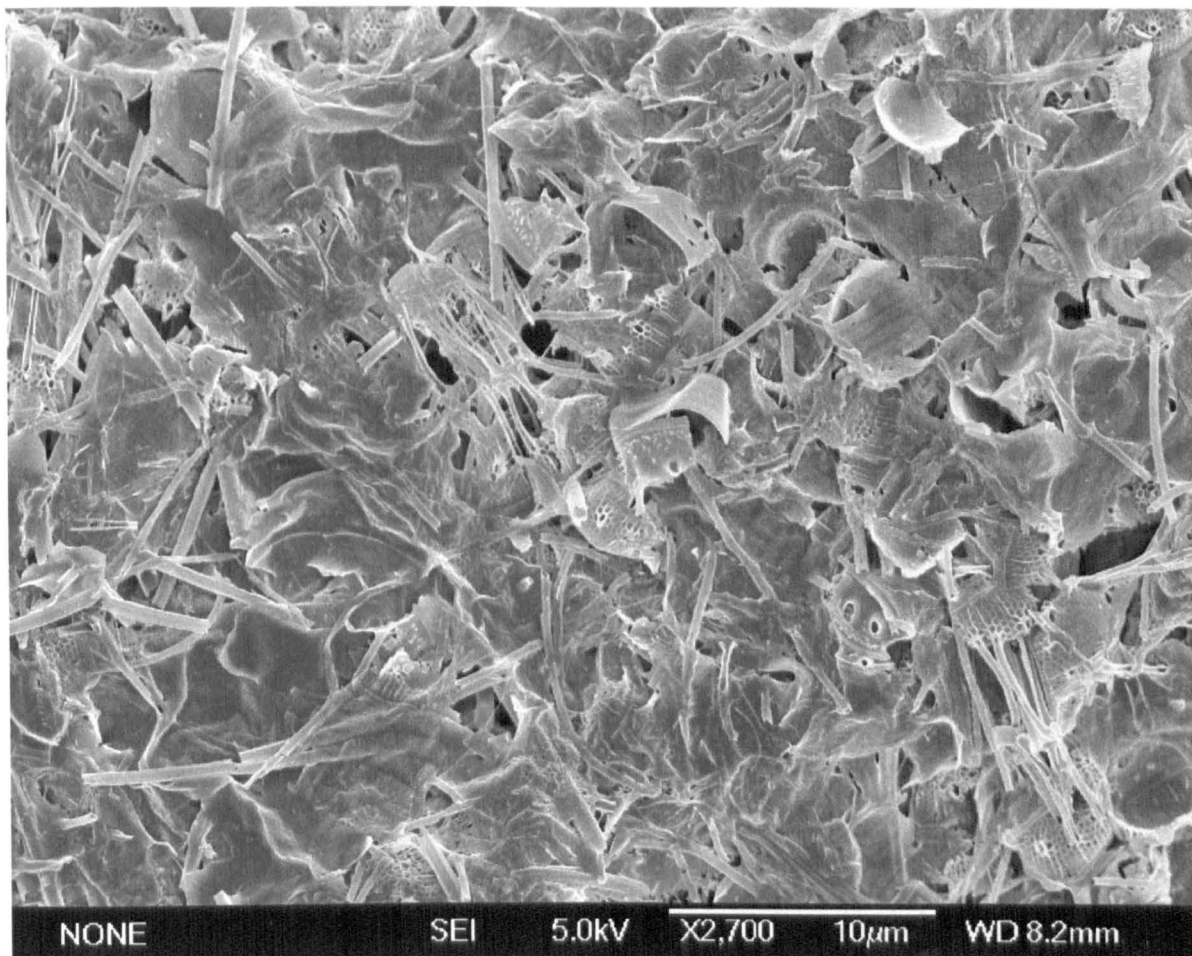
Figure 2-17: SEM photograph of a faecal pellet produced by a *Calanus helgolandicus* female collected at the beginning of the diatom bloom in 2009 (March 4<sup>th</sup>, station 2). The red circle shows an intact cell of *Skeletonema marinoi*.

At the beginning of the bloom (early March), copepods were evidently feeding on *S. marinoi* and although some cells seemed to pass intact through the gut (Figure 2-17), a large amount was digested, as demonstrated by the numerous fragments of *Skeletonema* cells observed in the samples. Contributions of other diatoms, such as *Chaetoceros spp.*, *Pseudo-nitzschia spp.*, *Thalassiosira rotula*, and unidentified pennate diatoms were minimal.



**Figure 2-18:** SEM photograph of a faecal pellet produced by a *Calanus helgolandicus* female collected on March 16<sup>th</sup> 2009 from station 2.

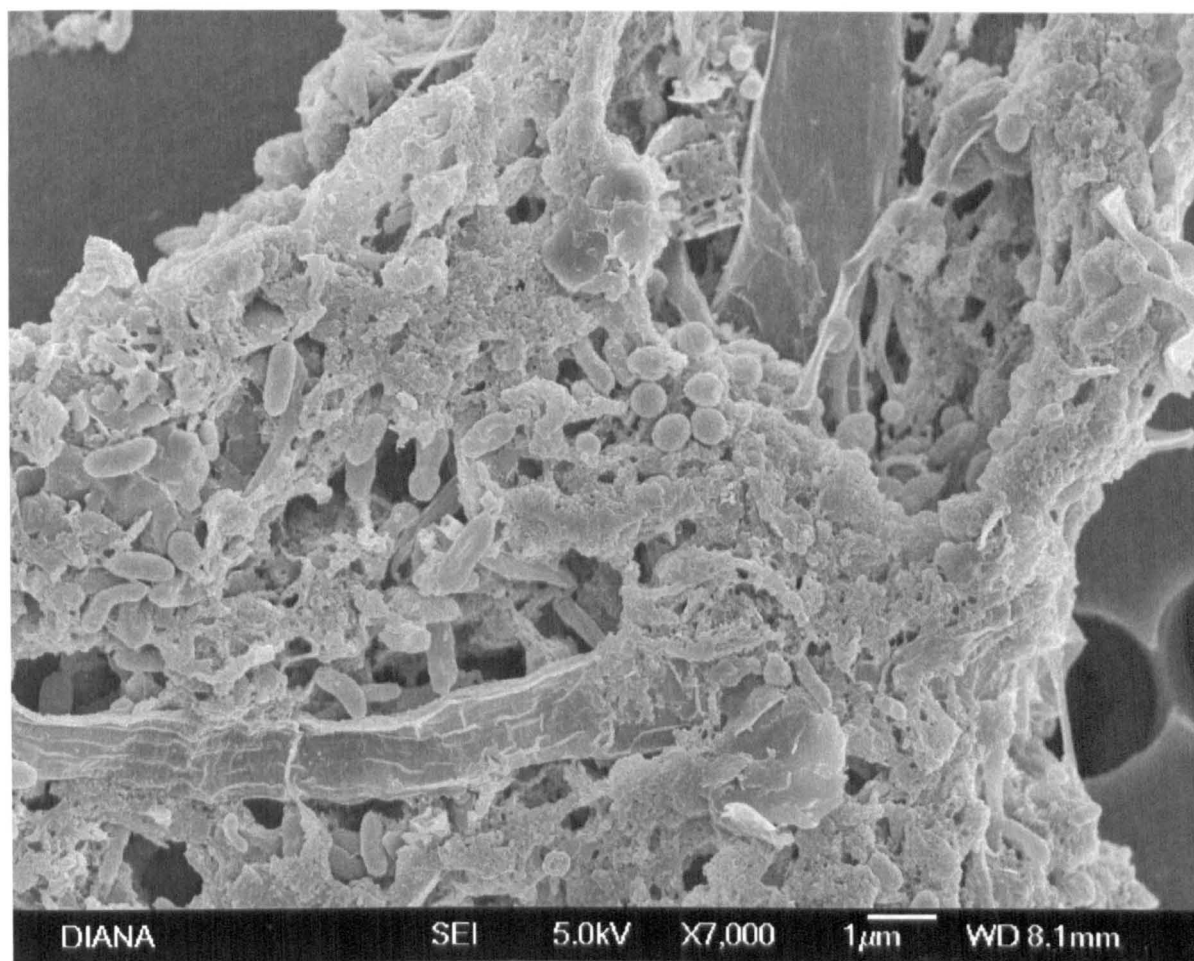
In samples collected during the bloom (March 16th), faecal pellets were almost entirely composed of *S. marinoi*. Interestingly, cells also seemed more digested (Figure 2-18). Cell fragments prevailed and few intact cells could be identified. There also seemed to be less unidentified amorphous material compared to the previous sample (Figures 2-17 and 2-18).



**Figure 2-19: SEM photograph of a faecal pellet produced by a *Calanus helgolandicus* female collected on March 24<sup>th</sup> 2009 from station 2.**

On March 24<sup>th</sup>, faecal pellet composition had changed. *S. marinoi* was still very abundant, but *Chaetoceros spp.* also contributed to faecal pellet composition (Figure 2-19). Cells of *Chaetoceros spp.* were largely digested, but their presence was noticeable due to the occurrence of spines in the pellets (Figure 2-19).





**Figure 2-20: SEM photograph of a faecal pellet produced by a *Calanus helgolandicus* female collected on March 30<sup>th</sup> 2009 from station 2.**

On March 30<sup>th</sup>, samples differed completely from previous samples. Some cell fragments still indicated feeding on *S. marinoi* and other phytoplankton were identifiable (e. g. *Emiliana huxleyi* and other coccolithophorids). However, faecal pellets seemed to be composed mainly of amorphous material with high amounts of bacteria (Figure 2-20). The bloom on this date had crashed (A. Miralto, pers. comm.) and copepods were probably feeding either on very digestible phytoplankton such as flagellates or on a scarce food supply, as suggested also by the low amount of faecal pellets produced.



## 2.4 Discussion

In 2005, egg production rates of *Calanus helgolandicus* remained constant in the Northern Adriatic Sea during spring. Egg production was not apparently negatively affected by the presence of oxylipin-producing diatoms during this time period. This corresponds to observations that diatoms can support high egg production rates, even though they may be deleterious to hatching success of the eggs produced (Ianora and Poulet 1993; Miralto *et al.* 2003; Vargas *et al.* 2006). However, neither was there an observable positive influence on egg production through increased food supply during the diatom bloom, since egg production rates remained unaffected by diatom abundance. Egg hatching success started to decrease at the end of February and continued decreasing with an increasing presence of diatoms at sea until reaching a low at the beginning of April with only ~20% of eggs developing to hatching. However, hatching success then started to recover while diatom numbers were still high.

Phytoplankton cell counts of collected net samples showed that *Skeletonema marinoi* was the main phytoplankton species present during the bloom and although other diatoms were contributing to the bloom at some stations, they consisted of species also known to produce oxylipins. Although there were slight variations in phytoplankton composition among stations, all phytoplankton samples collected consisted mainly of *S. marinoi*. Differences among stations were due to varying contributions of *Chaetoceros spp.* and *Pseudo-nitzschia spp.* Although *Chaetoceros* and *Pseudo-nitzschia* species have not been found to produce short-chain polyunsaturated aldehydes, they have been found to produce other oxylipins (Fontana *et al.* 2007b; d'Ippolito *et al.* 2009) that negatively impact copepod reproductive success (Miralto *et al.* 1999; Fontana *et al.* 2007b). Indeed, stations differed neither in oxylipin production of the phytoplankton assemblage, nor in hatching

success of copepod eggs. All stations seemed to have the same effect on copepod reproductive success throughout the course of the bloom.

Although copepods could have been feeding also on other sources such as microzooplankton (Calbet and Saiz 2005), *S. marinoi* strongly dominated the phytoplankton assemblage. It is likely, therefore, that copepods were feeding on *S. marinoi*. This was confirmed by SEM analyses of faecal pellets, which were carried out for a different year, but probably indicated the general feeding trend of *C. helgolandicus* during the spring diatom bloom in the Northern Adriatic Sea. Faecal pellet composition demonstrated that *S. marinoi* was grazed abundantly by *C. helgolandicus* during the bloom. Furthermore, cells were metabolized by copepods, showing that copepods were exposed to all of the metabolites released by cells during cell lysis (Pohnert 2000). It can therefore be assumed that copepods were indeed exposed to oxylipins during the spring diatom bloom in 2005.

Strangely, oxylipin production by the phytoplankton samples did not correlate to the presence of diatoms at sea, being higher during February than during the peak of the bloom. In terms of cellular production, the peak observed before the bloom seems to be some kind of signal, even though until now oxylipin cellular production has been suggested to be high at the end of blooms and not at the beginning (Vardi *et al.* 2006; Vidoudez and Pohnert 2008) (see Chapters 6 and 7). Oxylipins normalized for protein should be more indicative of potential oxylipin concentrations in the seawater and therefore should coincide with the presence of diatoms. However, also in this case higher oxylipin production could be observed at the beginning of the bloom, when diatom concentrations were low. Apparently, high cellular production of diatoms led to a general

increased potential for oxylipin production in the phytoplankton assemblage during February.

There was a delayed effect of oxylipins on egg hatching success. Aldehydes and other oxylipins were mainly produced in February and at the beginning of March, whereas egg hatching success was high during this time period and did not reach a minimum until the beginning of April, when oxylipin production in the phytoplankton assemblage was low. This may be due to a shift in effects with copepod females exposed to higher oxylipin concentrations during February and early March, which led to the production of eggs compromised for hatching success in the following weeks. Also in culture studies, it has been observed that several days to weeks are needed to induce a deleterious effect in hatching success when copepods are feeding on oxylipin-producing diatoms (Turner *et al.* 2001; d'Ippolito *et al.* 2002b; Ceballos and Ianora 2003). Apparently, the negative effect of diatoms on copepod reproduction can either be compensated by females for a short time period or accumulation processes are necessary to induce a deleterious effect on copepod egg development. This delayed effect of oxylipins on copepod reproduction is in accordance with the observed recovery of hatching success at the end of April, when oxylipin production in phytoplankton samples was low, even though diatom numbers were still high.

The decrease in hatching success showed a direct correlation to increasing diatom concentrations at sea, mostly due to *S. marinoi*, with hatching success compromised most strongly during the bloom period. This suggests an immediate impact of *S. marinoi* on copepod reproduction. However, this effect was apparently not mediated by the known end-metabolites of fatty acid oxidation, the oxylipins, because production was low during the peak of the bloom. Lipoxygenase (LOX) activity, as determined by the production of

fatty acid hydroperoxides (FAHs) with the colorimetric assay, on the other hand, showed a more direct correlation with *S. marinoi* concentrations at sea and therefore also with copepod egg hatching success. Production of FAHs increased with an increase of diatom concentrations, linking FAH production to the presence of diatoms in the sample. FAHs have been found themselves to be deleterious to hatching success and the effect described is actually stronger than that observed for aldehydes or other oxylipins (Fontana *et al.* 2007b). Therefore it is possible, that FAHs were directly impacting copepods during the diatom bloom, inducing an apoptotic effect in copepod eggs leading to decreased viability.

In comparison with the previous two years, the bloom of *S. marinoi* in 2005 had an intermediate effect on copepod reproduction. Hatching success was lower in 2003 which corresponded to a higher mean cellular oxylipin production; hence algae were more “toxic” with regards to oxylipin production. In 2004, phytoplankton was producing low amounts of oxylipins which corresponded to high hatching success. Although variations in oxylipin production may not correlate directly with hatching success within a diatom bloom, in years with more “toxic” algae, copepod reproduction is compromised more strongly than in years in which diatom cells are producing low amounts of fatty acid derived oxygenated metabolites. When oxylipin production is normalized for protein, mean yearly production of oxylipins does not coincide with the mean hatching success of that year. It therefore seems to be more important that diatom cells are producing high amounts of oxylipins than that the overall production of oxylipins in the total phytoplankton assemblage is high. As copepods always eat more or less the same amount of a diatom species, they are more strongly impacted in years with more “toxic” cells.

## 2.5 Conclusions

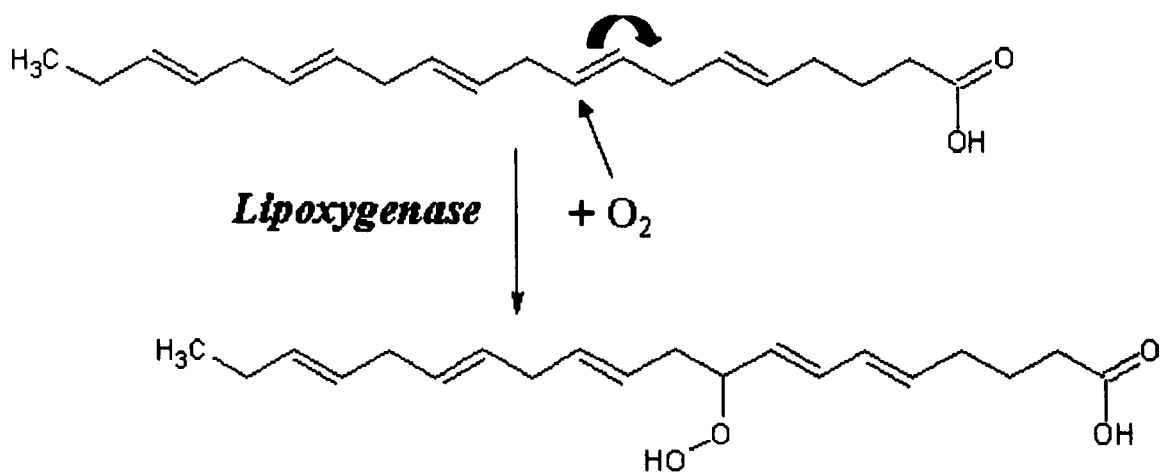
To conclude, hatching success of copepod eggs was strongly impacted during the height of the *Skeletonema marinoi*-dominated diatom bloom in 2005. This was possibly due to diatom oxylipin metabolism, since oxylipins were produced by the phytoplankton assemblage and copepods seemed to be feeding abundantly on *S. marinoi*. A decrease in hatching may have been caused either by a delayed effect of the more stable secondary oxylipins such as epoxyalcohols and hydroxy-acids produced at the beginning of the bloom, or by a more direct effect of the intermediate fatty acid hydroperoxides (FAHs) produced during the height of the bloom. It remains to be seen for what reason the production of secondary oxylipins such as aldehydes, epoxyalcohols and hydroxy-acids does not correspond to their presumed precursors, the FAHs. In any case, in years with stronger average oxylipin production by the phytoplankton assemblage, the average hatching success during the bloom period seems to be compromised more strongly than in years with weak oxylipin production.

### 3 Method development

Using the colorimetric assay for the determination of lipoxygenase (LOX) activity via the production of fatty acid hydroperoxides (FAHs) posed several problems, such as a lack of linearity and a lack of increase from blank to sample values. These problems were addressed analysing phytoplankton laboratory cultures and including a polarographic assay for measuring LOX activity. The lack of linearity in the colorimetric assay was ascribed to an inability of the secondary colorimetric reaction to accurately follow FAH production at high production rates. The polarographic assay showed improved linearity since it directly measured the consumption of one of the reactants. Furthermore, the colour compound formed in the colorimetric assay was found to be instable, making it imperative to read samples always at precise time intervals. The colorimetric assay was restricted to a pH-value of 6.0 and could not be adapted to the pH of seawater (8.15), an adaptation which was possible with the polarographic assay. The pH-value of the buffer was not only important for LOX activity measurements, but also seemed important for the extraction of samples for oxylin quantification with lower oxylin production determined at lower pH-values. Freezing samples had no significant influence on the two LOX assays and on oxylin production and could therefore be applied to the collection of field samples.

### 3.1 Introduction

All phytoplankton samples collected in the field were analyzed for lipoxygenase (LOX) activity by the colorimetric assay and extracted with organic solvents for identification and quantification of oxylipins via mass spectrometric analyses. However, several difficulties were encountered using the colorimetric assay, which were addressed by analyzing samples obtained from phytoplankton laboratory cultures. First of all, there seemed to be a problem with the linearity of the assay. Adding twice the sample amount in the assay (8 mg instead of 4 mg wet weight) never led to twice the absorbance. Another problem encountered in some cases was a lack of increase in absorbance from the blank to the sample, and also a lack of increase in absorbance upon the addition of exogenous fatty acids, which should have increased fatty acid hydroperoxide (FAH) production by LOX. Even though samples were producing oxylipins, LOX activity could not be demonstrated by the colorimetric assay in these cases. Therefore in this chapter another assay was examined for its applicability to measuring LOX activity. This assay is based on the consumption of dioxygen during the oxidation of fatty acids to FAHs by LOX enzymes, which should take place in a molar ratio of 1:1 (Figure 3-1).



**Figure 3-1: Oxidation of polyunsaturated fatty acids to fatty acid hydroperoxides by lipoxygenase via insertion of a dioxygen molecule.**

Oxygen consumption rate was measured polarographically in an oxygraph and related to LOX activity (Axelrod *et al.* 1981). While the colorimetric assay was normally carried out at pH=6.0 (pH-range: 5.5-7.0 (Anthon and Barrett 2001)), the oxygraph could be used under various pH-conditions, because it directly measures the rate of oxygen consumption, independent of a secondary reaction. To create a similar environment as presumably encountered by LOX enzymes at sea, LOX activity in the cell lysate was generally determined in buffer at the pH of seawater (8.15) in the polarographic assay. Therefore it was examined whether the colorimetric assay could also be adapted to this pH.

Since analysis of LOX activity was carried out on field phytoplankton samples that had been frozen in liquid nitrogen and kept at -80°C until analysis, studies in this chapter also established whether this handling of the samples could influence the results.



## 3.2 Materials and Methods

### 3.2.1 Phytoplankton Culture Samples

Phytoplankton cultures were grown in two-litre polycarbonate bottles under gentle bubbling with sterile (0.22- $\mu\text{m}$  filtered) ambient air. Cultures were kept in a climate chamber at 20°C on a 12h:12h light:dark cycle at 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Samples were harvested in stationary phase by centrifugation in a cooled centrifuge (DR 15P, Braun Biotechnology International) with a swing-out rotor (Table 3-1).

For comparative analyses of fresh and frozen samples, half of the culture was centrifuged and analysed immediately, whereas the other half was pelleted, frozen in liquid nitrogen and kept at -80°C until analysis in analogy to field phytoplankton samples. Four replicates each of *Chaetoceros affinis* and the *Skeletonema marinoi* clone isolated in 2003 from the Northern Adriatic Sea were analysed in a fresh and frozen state. The same isolate of *S. marinoi* was used in all other methodological analyses, except for the pH-comparisons, which were carried out with an isolate from 1997 for lipoxygenase (LOX) activity and aldehyde production and with a clone isolated in 2004 for production of oxylipins other than aldehydes. Selection of clones depended on availability at the moment of analysis.

Phytoplankton cultures were concentrated by centrifugation in a pre-weighed blue Falcon tube (BectonDickinson) and the total volume was brought to 50 ml before final centrifugation. From this volume, a 100  $\mu\text{l}$  subsample was taken to determine cell counts. The cell count subsample was diluted 1:30 with filtered seawater, fixed with two drops of Lugol's solution and counted in a Sedgewick counting chamber (Hausser Scientific, Horsham, PA, USA) under an inverted microscope (10x). After the last centrifugation step,

a quick-spin (30 s) of the pellet at increased rotational speed was carried out (Table 3-1) to remove excess water. The pellet weight was determined before analysis or freezing.

**Table 3-1: Centrifugation parameters for collection of culture pellet samples of three diatom species *Chaetoceros affinis*, *Skeletonema marinoi*, and *Thalassiosira rotula*, used in this and the following chapter.**

Phytoplankton Species	Time [min]	Rot. Speed [×g]	Quick-spin [×g]	Temp. [°C]
<i>Chaetoceros affinis</i>	15	300	1900	18
<i>Skeletonema marinoi</i>	10	1000	2800	4
<i>Thalassiosira rotula</i>	10	1200	2800	4

**3.2.2 Polarographic Assay**

Oxygen consumption rate of the cell lysate was measured in a Gilson 5/6 oxygraph (Gilson Medical Electronics, Middleton, WI, USA) in a water-jacketed reaction vessel of two millilitre volume with a Clark electrode covered with a Teflon® membrane. The reaction vessel was kept at a constant temperature of 22°C by a circulating water bath. Measurements were conducted in 0.2 M sodium phosphate buffer at pH=6.0 or in 0.2 M sodium borate buffer at pH=8.15. 1.9 ml of buffer was left to equilibrate with atmospheric oxygen in the open reaction vessel for 5 min, after which the vessel was closed with a capillary bore stopper and the blank was registered for 5 min. The cell lysate (5-70 µl) was added by an automatic pipette and oxygen consumption rate was registered for another 5 min. Following the addition of eicosapentaenoic acid (EPA) (0.2 or 0.4 mM final concentration; solution prepared as in 2.2.3), oxygen consumption rate was registered for an additional 5 min. Aliquots of samples were added according to the aliquots used in the

colorimetric assay corresponding to 4 mg of sample wet weight and multiples thereof. Oxygen consumption rate was recorded by a writer head on heat-sensitive millimetre paper moving at a constant speed. Calculations were carried out by comparing the slopes of oxygen consumption rates from the linear part of the curves. Lipoxygenase (LOX) activity determined polarographically was normalized for protein content which was measured as described in Chapter 2 (2.2.6).

The specificity of the polarographic assay for determining LOX activity in phytoplankton lysates was evaluated as follows. The increase in oxygen consumption rate upon addition of exogenous fatty acid (EPA) to two species known to lack LOX activity, the flagellate *Prorocentrum minimum* (Fontana *et al.* 2007b) and the diatom *Thalassiosira weissfloggii* (Wichard *et al.* 2005; Wichard *et al.* 2007), was compared to the increase in oxygen consumption rate in *Skeletonema marinoi*, known to possess LOX activity (Fontana *et al.* 2007b).

### 3.2.3 Colorimetric Assay

Lipoxygenase (LOX) activity was determined colorimetrically as described in Chapter 2 (2.2.3). In the present study, however, LOX activity was calculated from the difference in absorbance between the blank and the sample. LOX activity was normalized for protein. Alternatives to sodium dodecyl sulphate (SDS) for blocking LOX enzymes before reading the absorbance in the spectrophotometer were also tested.

The specificity of the colorimetric assay for determining LOX activity was evaluated as follows. Pure LOX enzyme (3.2.5) was incubated with eicosapentaenoic acid (EPA) after the addition of the LOX inhibitor nordihydroguaiaretic acid (NDGA) at 50  $\mu$ M.

### 3.2.4 Oxylipin Analysis

Production of volatile aldehydes and oxylipins other than aldehydes was determined as described in Chapter 2 (2.2.4) and was normalized for cells. For the analysis of fresh and frozen samples, the production of aldehydes and oxylipins other than aldehydes was pooled to compare oxylipin production between treatments.

### 3.2.5 Calibration

For calibration purposes, a solution of pure lipoxygenase (LOX) enzyme (lipoxidase from soybean at 45,000 units  $\text{mg}^{-1}$ ) was prepared in 0.2 M sodium borate buffer (pH=8.15) at 2  $\text{mg ml}^{-1}$ . This solution was also used for some of the methodological studies. In the colorimetric and polarographic assays, aliquots of LOX enzyme were incubated at different concentrations with eicosapentaenoic acid (EPA) at 0.4 mM final concentration according to 2.2.3 and 3.2.2.

## 3.3 Results

### 3.3.1 Specificity of Lipoxygenase Activity Assays

#### 3.3.1.1 Polarographic Assay

In *Thalassiosira weissflogii*, oxygen consumption rate of the cell lysate was low and did not increase upon the addition of eicosapentaenoic acid (EPA) (Figure 3-2). Also in *Prorocentrum minimum*, oxygen consumption rate did not increase upon EPA-addition. In *Skeletonema marinoi* lysate on the other hand, oxygen consumption increased significantly upon the addition of exogenous EPA (t-test:  $t=5.18$ ,  $df=11$ ,  $p=0.0003$ ). This activity could

be blocked completely when a boiled cell lysate of *S. marinoi* was used in the assay (Figure 3-2).

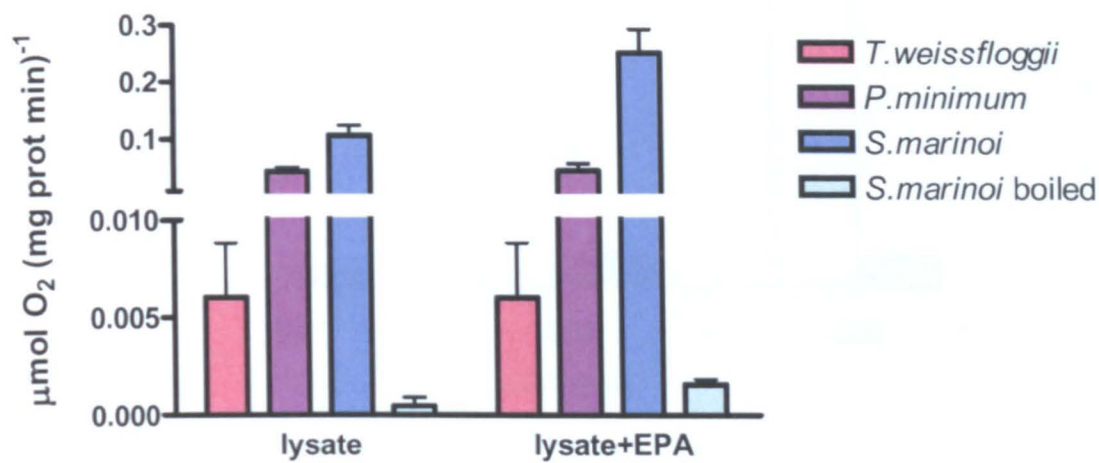
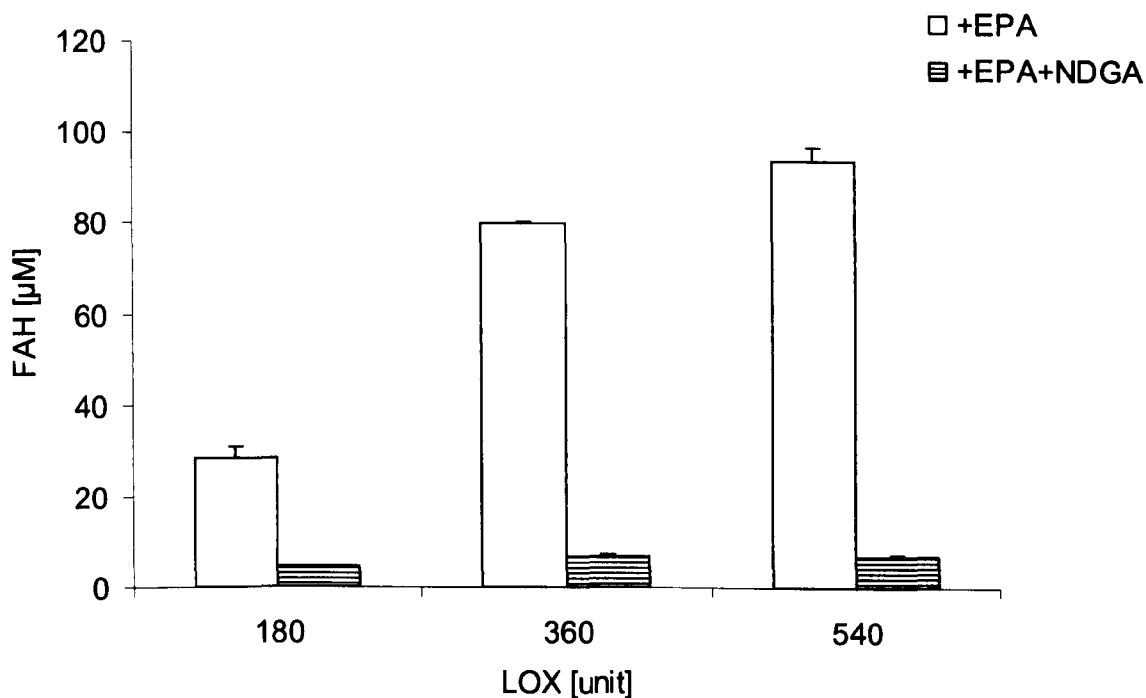


Figure 3-2: Mean oxygen consumption rate (+SEM, n=2 except *Skeletonema marinoi*: n=12) [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] in cell lysate (lysate) and in cell lysate incubated with eicosapentaenoic acid (0.2 mM) (lysate+EPA) of *Thalassiosira weissfloggii*, *Prorocentrum minimum*, and *S. marinoi*, including a boiled sample of *S. marinoi* measured with the polarographic assay at pH=8.15.

3.3.1.2 Colorimetric Assay

Preliminary studies with the lipoxygenase (LOX) inhibitor nordihydroguaiaretic acid (NDGA) indicated that fatty acid hydroperoxide (FAH) production was indeed due to LOX activity because NDGA completely blocked FAH production (Figure 3-3). Inhibitor studies were then abandoned because NDGA failed to block activity of both cell lysate and pure LOX enzyme from soybean (3.2.5) at pH=8.15 in the polarographic assay.

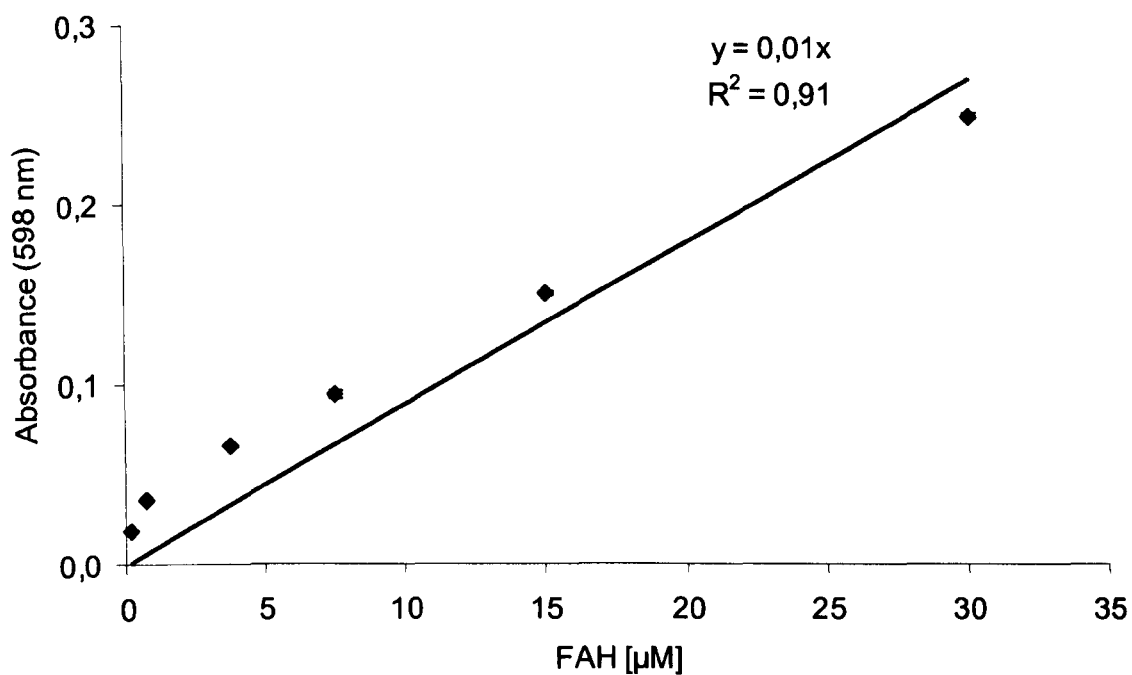


**Figure 3-3: Mean fatty acid hydroperoxide (FAH) production (+SEM, n=2) [μM] from varying amounts of pure lipoxygenase (LOX) enzyme [unit] incubated with 0.4 mM eicosapentaenoic acid only (+EPA) and with EPA and the LOX inhibitor (0.05 mM) nordihydroguaiaretic acid (+EPA +NDGA) determined with the colorimetric assay.**

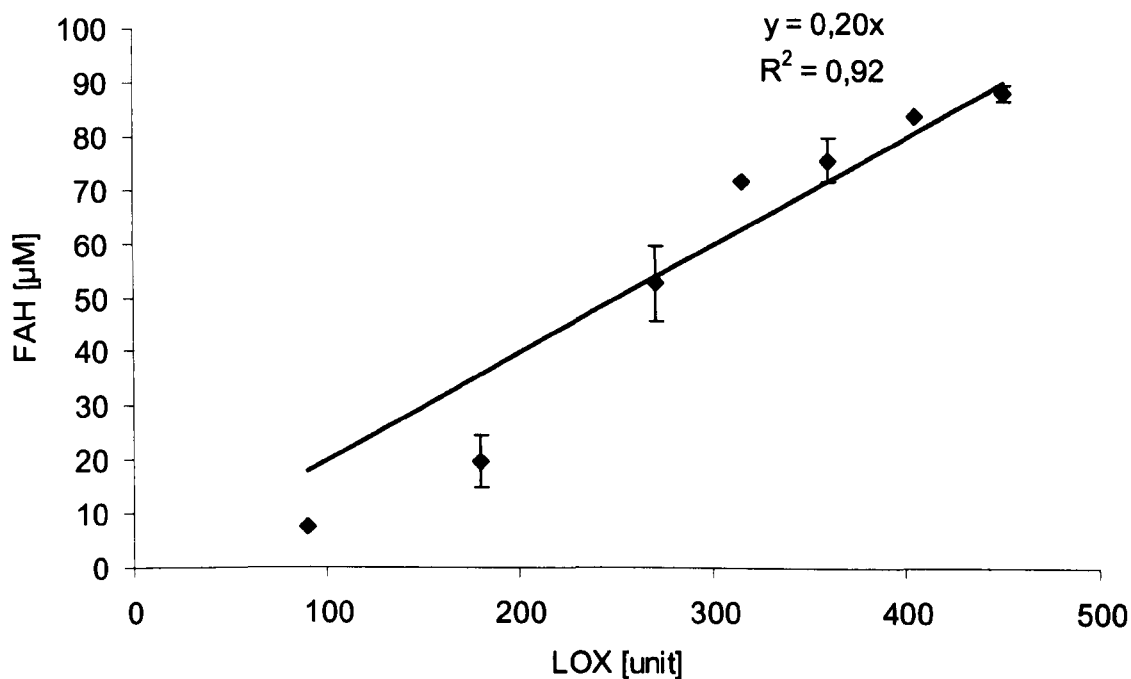
### 3.3.2 Linearity

#### 3.3.2.1 Colorimetric Assay

When the colorimetric assay was calibrated directly with eicosapentaenoic acid hydroperoxide (FAH), the relationship between FAH concentration and absorbance at 598 nm remained linear up to at least 30 μM (Figure 3-4). This corresponded to the concentration range given for linearity in the original paper by Anthon and Barrett (2001). However, FAH concentrations produced by phytoplankton lysates were often higher than this value.



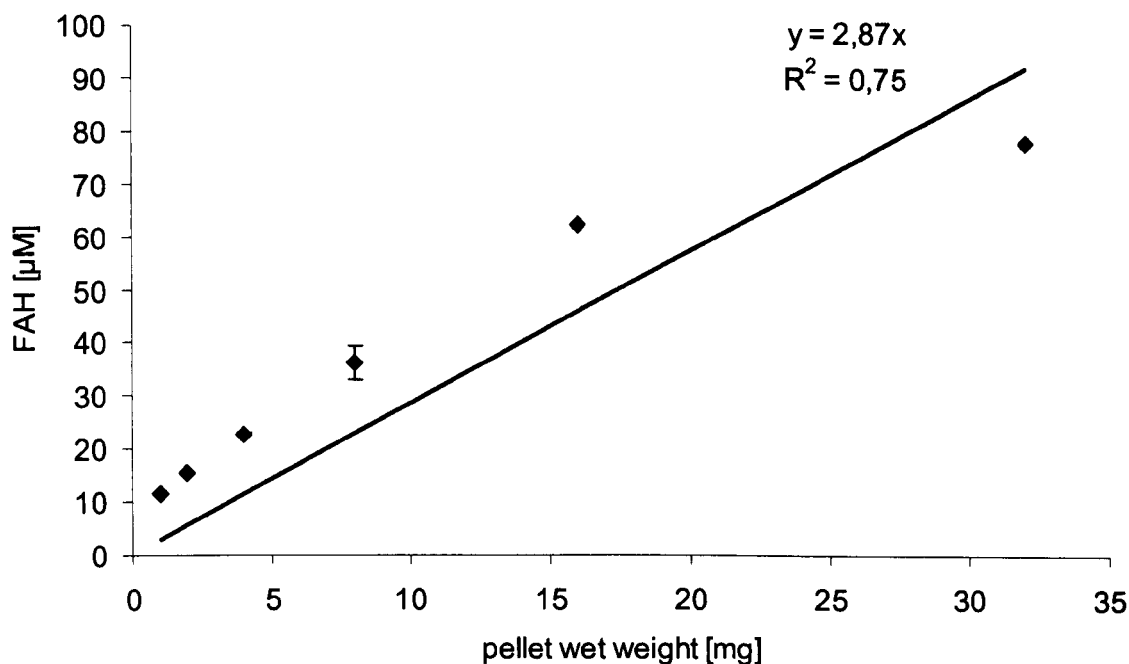
**Figure 3-4: Relationship between mean absorbance ( $\pm\text{SEM}$ ,  $n=2$ ) at 598 nm and concentration of eicosapentaenoic acid hydroperoxide (FAH) [ $\mu\text{M}$ ] in the colorimetric assay.**



**Figure 3-5: Relationship between mean fatty acid hydroperoxide (FAH) concentration ( $\pm$ SEM,  $n=1-3$  depending on LOX amount) [ $\mu$ M] and amount of lipoxygenase (LOX) [unit] incubated with eicosapentaenoic acid (0.4 mM) in the colorimetric assay.**

When the assay was calibrated with pure lipoxygenase (LOX) enzyme with eicosapentaenoic acid (EPA) as substrate (0.4 mM final concentration), linearity was in fact restricted to a LOX concentration below 450 units, after which the curve levelled off (Figure 3-5). However, compared to incubating directly with the hydroperoxide, absorbance seemed to be linear to LOX amount up to a concentration of  $\sim 90 \mu$ M FAH (Figure 3-5).



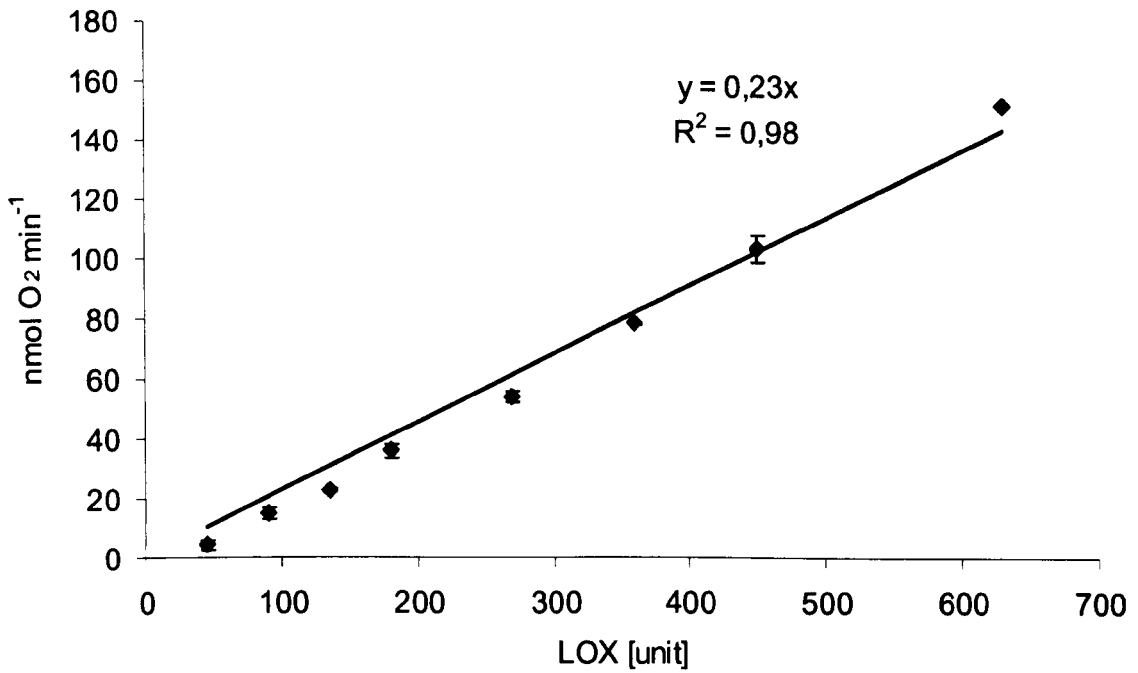


**Figure 3-6: Relationship between mean fatty acid hydroperoxide (FAH) concentration ( $\pm$ SEM,  $n=1-2$  depending on pellet wet weight) [ $\mu$ M] and *Skeletonema marinoi* pellet wet weight [mg] in the colorimetric assay.**

When the colorimetric assay was carried out with a cell lysate of *Skeletonema marinoi*, the relationship between the amount of algal sample added and the amount of FAH detected was linear up to  $\sim 16$  mg wet weight of sample which corresponded to a FAH concentration of  $\sim 60 \mu$ M (Figure 3-6).

### 3.3.2.2 Polarographic Assay

The polarographic assay showed greater linearity when calibrated with pure lipoxygenase (LOX) enzyme and eicosapentaenoic acid (EPA) (0.4 mM final concentration) than the colorimetric assay (Figure 3-7). The relationship between LOX amount and oxygen consumption rate was linear up to the highest amount tested (630 units).

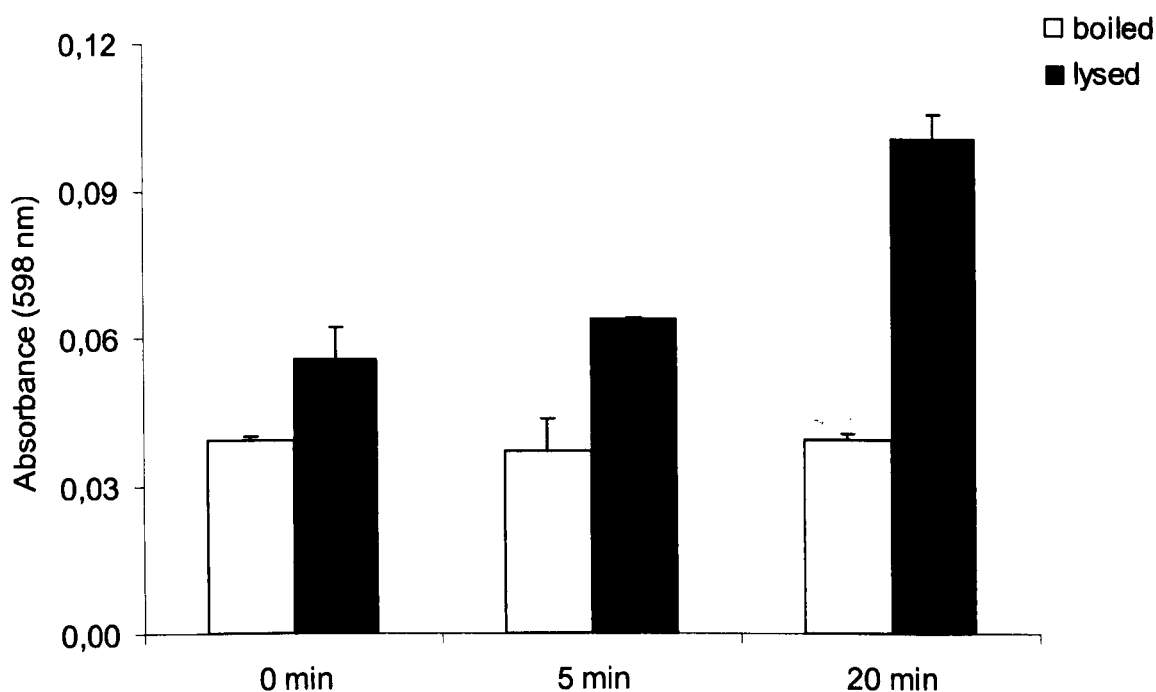


**Figure 3-7: Relationship between mean oxygen consumption rate ( $\pm$ SEM, n=1-3 depending on LOX amount) [nmol O<sub>2</sub> min<sup>-1</sup>] and amount of lipoxygenase (LOX) [unit] incubated with eicosapentaenoic acid (0.4 mM) in the polarographic assay at pH=8.15.**

### 3.3.3 Stopping the Colorimetric Assay

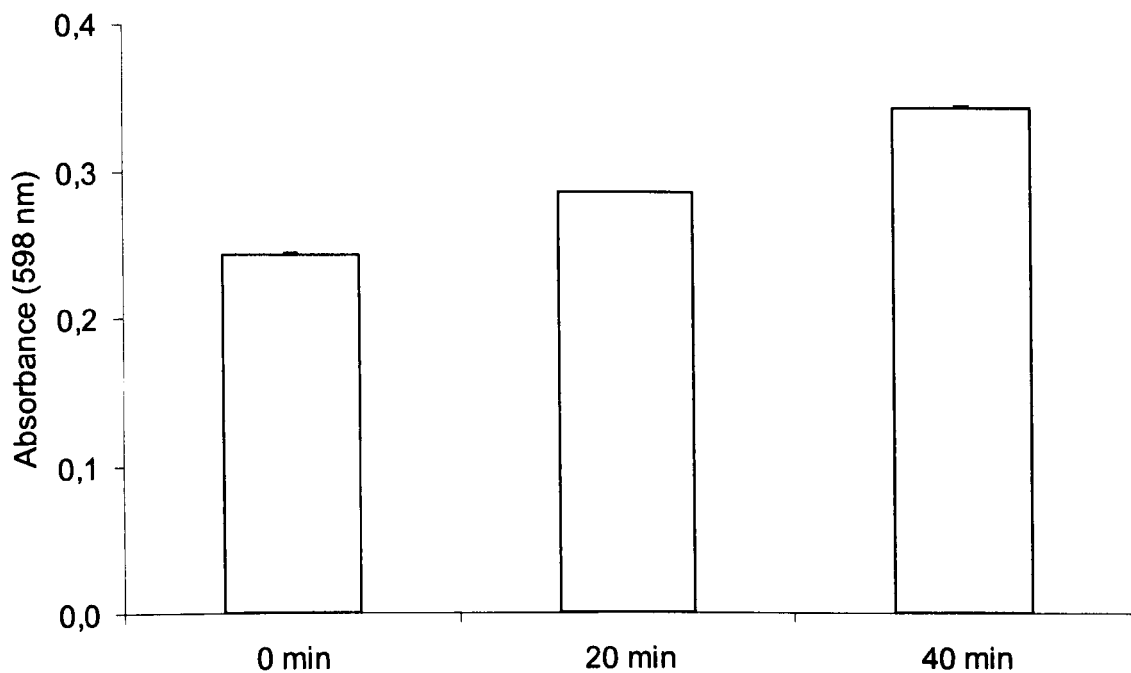
One alternative tested for stopping the LOX reaction before reading the absorbance of the sample was the addition of methanol after 20 min. This, however, led to conflicting results.

When samples were boiled prior to sonication, thereby prior to preparation of the cell lysate, *Skeletonema marinoi* cell lysate showed no increase in FAH production with time in contrast to a normally lysed cell suspension (Figure 3-8).

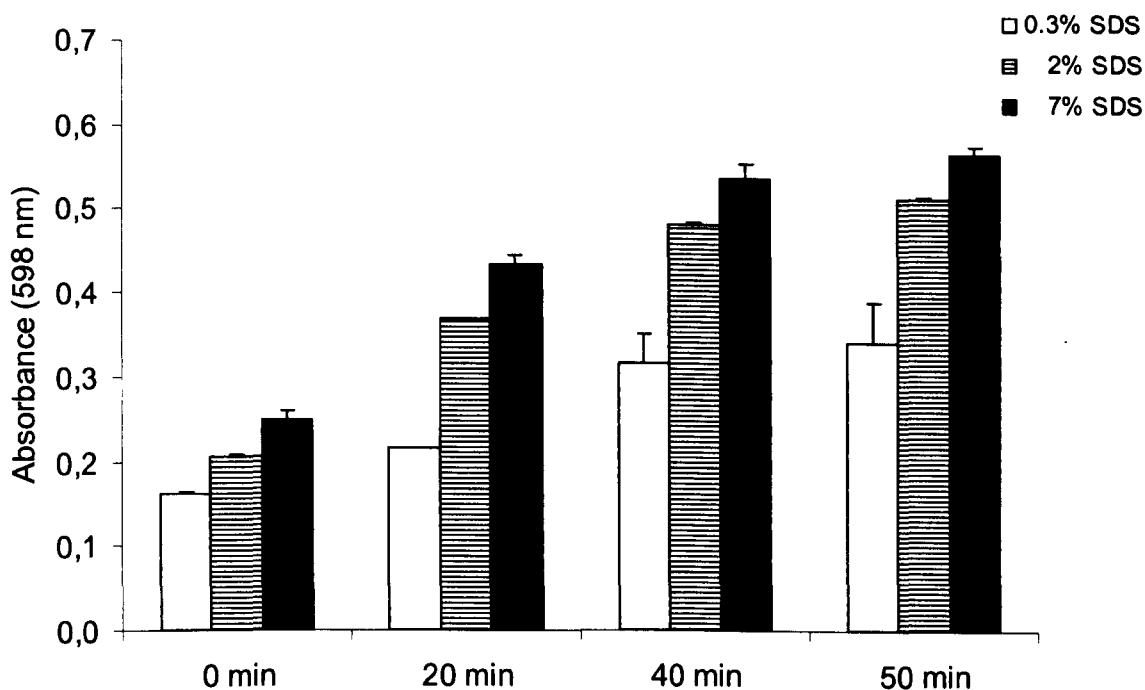


**Figure 3-8: Mean absorbance (+SEM, n=2) at 598 nm of a boiled cell lysate and a normally lysed cell suspension of *Skeletonema marinoi* at 0, 5, and 20 min after the start of the colorimetric assay.**

However, when boiling was used as a method to stop the reaction after 20 min prior to reading sample absorbance, a continued increase in colour formation was observed (Figure 3-9).



**Figure 3-9: Mean absorbance (+SEM, n=2) at 598 nm of samples read 0, 20, and 40 min after stopping the colorimetric assay by boiling samples. The colorimetric assay was carried out with cell lysate of *Skeletonema marinoi*.**



**Figure 3-10: Mean absorbance (+SEM, n=2) at 598 nm of samples read 0, 20, 40, and 50 min after stopping the colorimetric assay by addition of increasing concentrations of SDS (0.3, 2, and 7%). The colorimetric assay was carried out with a cell lysate of *Skeletonema marinoi*.**

The same steady increase in absorbance was demonstrated when SDS-concentrations for stopping the reaction after 20 min were increased (Figure 3-10). Even when the highest possible concentration of SDS (7%) was used, re-reading the samples after 20, 40, and 50 min after the initial reading led to a continuous increase in absorbance. However, it could be demonstrated polarographically that the same sample analyzed in buffer containing even SDS concentrations as low as 0.3% failed to show LOX activity.  $\text{Na}_2\text{SO}_3$  was added to the oxygraph to control that oxygen measurements were not influenced by the detergent itself and that the lack of registered oxygen consumption was actually due to an inhibitory effect of the detergent on LOX activity.

3.3.4 pH-variability

3.3.4.1 Colorimetric Assay

Carrying out the colorimetric assay with a cell lysate of *Skeletonema marinoi* at increasing pH led to a decrease in absorbance (Figure 3-11).

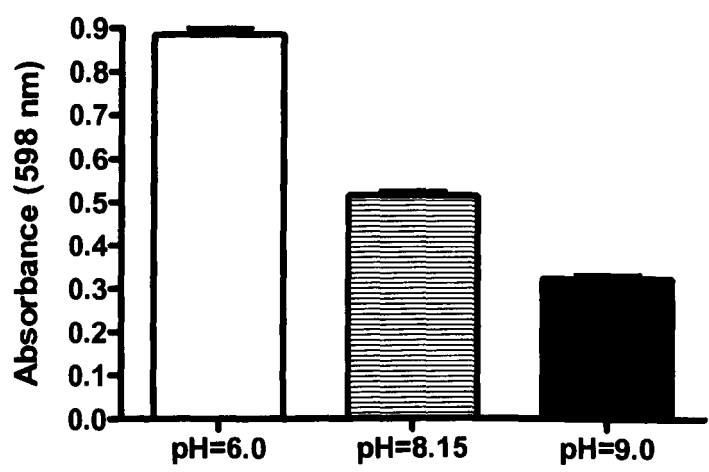
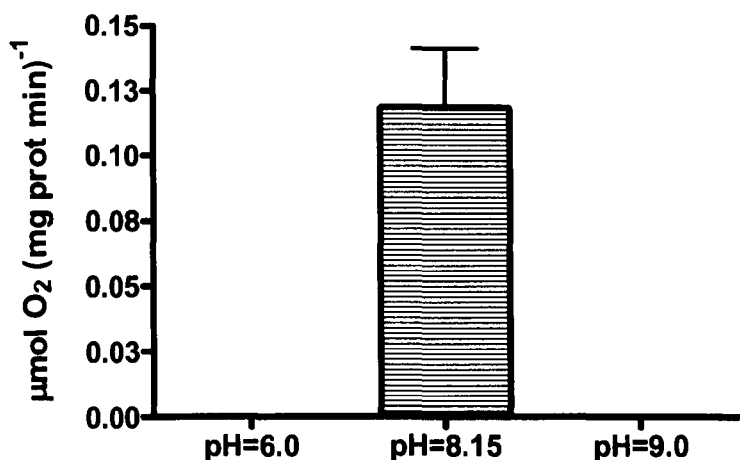


Figure 3-11: Mean absorbance (+SEM, n=2) at 598 nm of a cell lysate of *Skeletonema marinoi* measured in the colorimetric assay carried out at various pH (pH=6.0, 8.15, and 9.0). Absorbance values were not corrected for blank values.

3.3.4.2 Polarographic Assay

The same cell lysate of *Skeletonema marinoi* measured in the colorimetric assay at increasing pH was measured at the same pH-values in the oxygraph (Figure 3-12).



**Figure 3-12:** Mean oxygen consumption rate (+SEM; pH=6.0: n=2, pH=8.15: n=4, pH=9.0: n=1) [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] of *Skeletonema marinoi* cell lysate upon EPA-addition (0.2 mM) determined polarographically at various pH (pH=6.0, 8.15, and 9.0).

This demonstrated that apparently lipoxygenase (LOX) enzymes in this sample were not active with eicosapentaenoic acid (EPA) as substrate at pH=6.0 and pH=9.0. An increase in oxygen consumption rate upon the addition of EPA, hence LOX activity, was observed only at pH=8.15 (Figure 3-12).

#### 3.3.4.3 Oxylin Production

This pH-dependency of the lipoxygenase (LOX) reaction could also be demonstrated by extracting a cell lysate of *Skeletonema marinoi* suspended in buffer at two different pH (pH=6.0 and 8.15) and quantifying oxylin production according to 2.2.4 (Table 3-2).

**Table 3-2: Production of aldehydes and oxylipins other than aldehydes (n=1) normalized for cells [fg cell<sup>-1</sup>] in cell lysate of *Skeletonema marinoi* suspended in buffer at pH=6.0 and pH=8.15. Analysis of aldehyde production was carried out on a different sample of *S. marinoi* cell lysate than analysis of production of oxylipins other than aldehydes.**

pH	Aldehydes [fg cell <sup>-1</sup> ]	Other oxylipins [fg cell <sup>-1</sup> ]
6.0	145	13
8.15	238	127

Whereas aldehyde production seemed only weakly affected by pH, production of oxylipins other than aldehydes seemed to be strongly influenced by changes in pH-value. Aldehyde production increased only by ~65% when cell lysate was incubated in buffer at pH=8.15 instead of at pH=6.0. The production of other oxylipins, on the other hand, showed a ten-fold increase when cell lysate was incubated at pH=8.15 instead of pH=6.0. Low production of other oxylipins compared to aldehydes may be due to the fact that analyses for the two oxylipin groups were carried out on separate samples of *S. marinoi* and were therefore not comparable.

### 3.3.5 Comparison of Fresh and Frozen Phytoplankton Samples

#### 3.3.5.1 Colorimetric Assay

Production of fatty acid hydroperoxides (FAHs) in cell lysates of frozen samples was not significantly different from FAH production in cell lysates of fresh samples of *Chaetoceros affinis* (t-test:  $p>0.05$ ) (Figure 3-13).



***C.affinis***

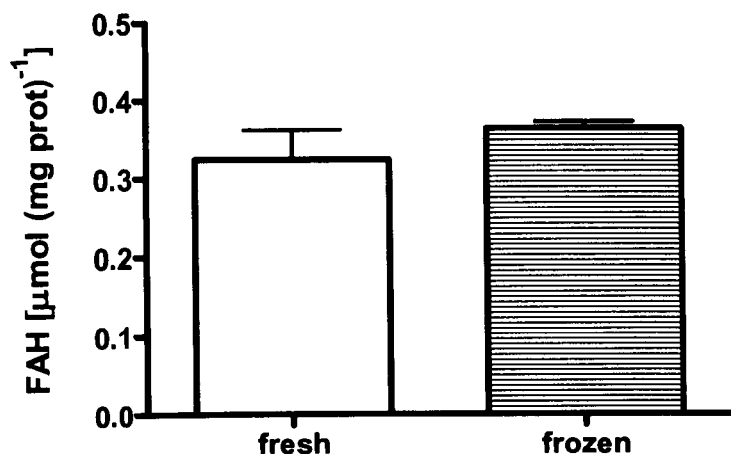


Figure 3-13: Mean production (+SEM, n=4) of fatty acid hydroperoxide (FAH) normalized for protein [ $\mu\text{mol (mg prot)}^{-1}$ ] in fresh and frozen samples of *Chaetoceros affinis*. Detection of FAH was carried out with the colorimetric assay.

***S. marinoi***

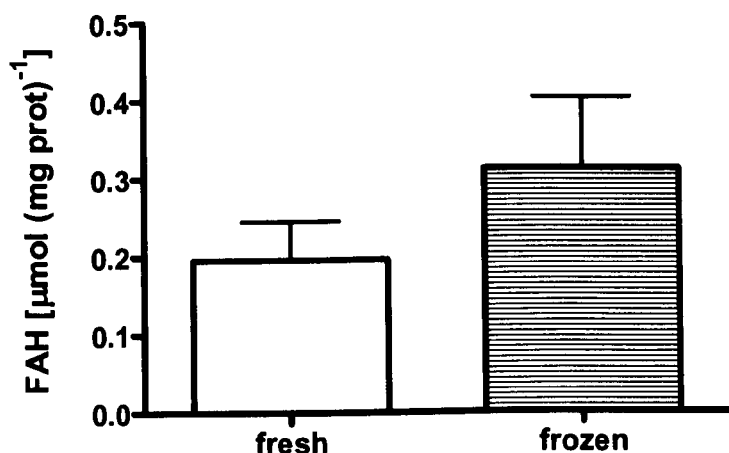


Figure 3-14: Mean production (+SEM, n=4) of fatty acid hydroperoxide (FAH) normalized for protein [ $\mu\text{mol (mg prot)}^{-1}$ ] in fresh and frozen samples of *Skeletonema marinoi*. Detection of FAH was carried out with the colorimetric assay.

Also for *Skeletonema marinoi*, cell lysate of frozen samples did not produce significantly higher concentrations of FAHs than cell lysate of fresh samples ( $t=2.5$ ,  $df=3$ ,  $p>0.05$ ) (Figure 3-14).

3.3.5.2 Polarographic Assay

Lipoxygenase (LOX) activity determined polarographically at pH=8.15 was not significantly different between fresh and frozen samples of *Chaetoceros affinis* (t-test:  $p>0.05$ ) (Figure 3-15).

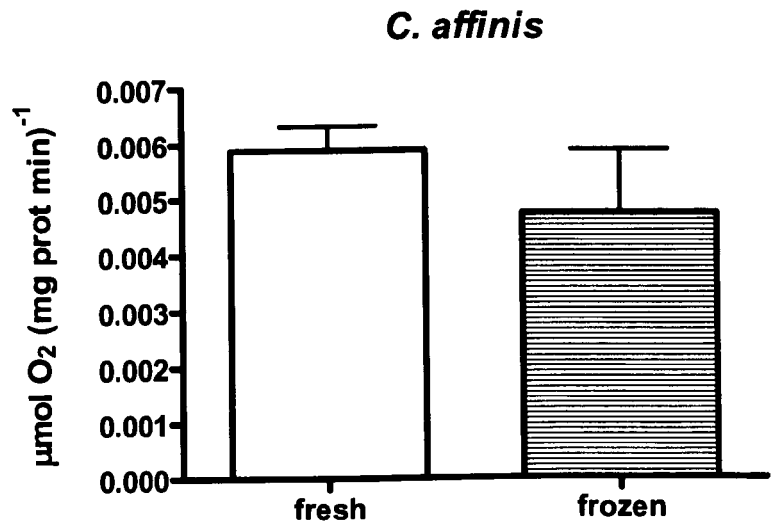
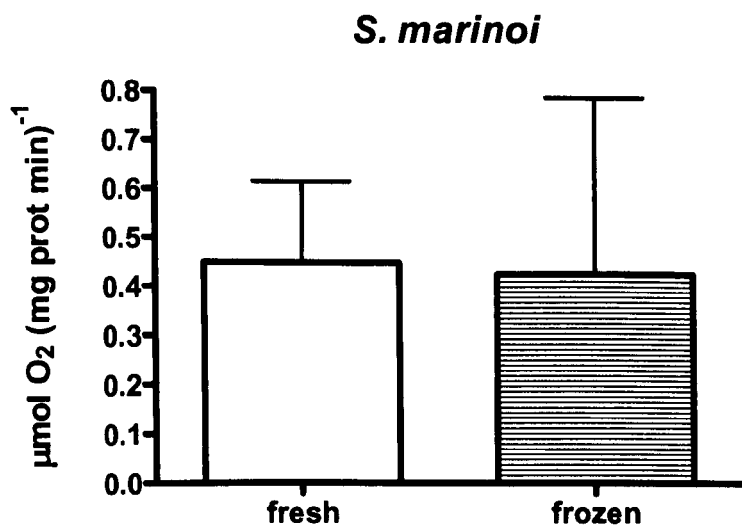


Figure 3-15: Mean oxygen consumption rate (+SEM, n=4) [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] upon EPA-addition determined polarographically in fresh and frozen samples of *Chaetoceros affinis* at pH=8.15.

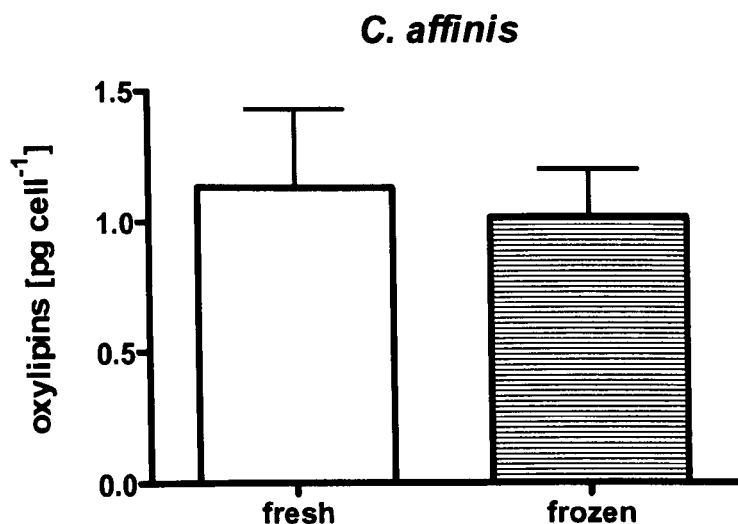


**Figure 3-16: Mean oxygen consumption rate (+SEM, n=4) [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] upon EPA-addition determined polarographically in fresh and frozen samples of *Skeletonema marinoi* at pH=8.15.**

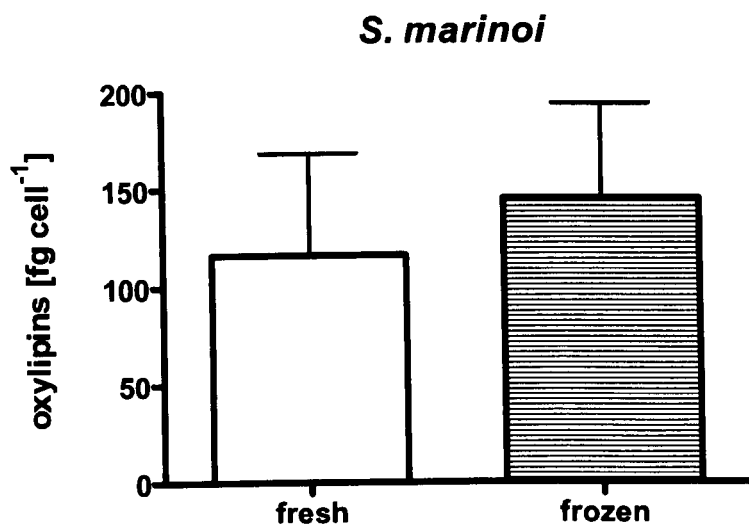
Differences between fresh and frozen samples in regard to LOX activity determined polarographically were also not observed in *Skeletonema marinoi* (t-test:  $p>0.05$ ) although variability of replicates in this species was very high (Figure 3-16).

### 3.3.5.3 Oxylipin Production

Cell lysates of fresh and frozen samples did not differ significantly in oxylipin production, neither when *Chaetoceros affinis* nor when *Skeletonema marinoi* were concerned (t-test:  $p>0.05$ ) (Figures 3-17 and 3-18). Representative chromatograms of GC- and LC-MS analyses of fresh and frozen samples of *C. affinis* and *S. marinoi* are to be found in Appendix 2.



**Figure 3-17: Mean oxylipin production (+SEM, n=4) normalized for cells [pg cell<sup>-1</sup>] in fresh and frozen samples of *Chaetoceros affinis*.**



**Figure 3-18: Mean oxylipin production (+SEM, n=4) normalized for cells [fg cell<sup>-1</sup>] in fresh and frozen samples of *Skeletonema marinoi*.**

Protein content remained the same in fresh and frozen samples, also indicating that enzymes were not significantly compromised during the freezing process.

### 3.4 Discussion

The colorimetric assay could be successfully employed for detecting lipoxygenase (LOX) activity through the production of fatty acid hydroperoxides (FAHs) in fresh, as well as in frozen samples of phytoplankton culture. Also the polarographic assay was specific for LOX activity when external fatty acids, e.g. eicosapentaenoic acid (EPA) were added to the cell lysate in the oxygraph. Only in the algal species known to display oxylipin metabolism, *Skeletonema marinoi*, was there an increase in oxygen consumption rate upon EPA-addition to the cell lysate, whereas a cell lysate of *Thalassiosira weissflogii* and *Prorocentrum minimum*, both known to lack oxylipin metabolism, showed no increase in oxygen consumption rate when EPA was added in the polarographic assay. The increase in oxygen consumption rate in *S. marinoi* cell lysate disappeared upon boiling, indicating the involvement of enzymes. The high oxygen consumption rate of the *S. marinoi* cell lysate without EPA also indicated LOX activity on endogenous fatty acids, which also disappeared with boiling. These results may suggest that the polarographic assay was specific for LOX activity and that activity could be defined as the difference between oxygen consumption rate of the cell lysate and oxygen consumption rate upon the addition of exogenous fatty acid to the cell lysate. Also in the colorimetric assay, it is possible to define LOX activity as the difference in FAH concentrations in the cell lysate incubated with and without the addition of exogenous fatty acids. However, in the colorimetric assay, LOX activity can also be defined as the difference in FAH concentrations between the cell lysate sample and the blank. As diatoms contain large reserves of polyunsaturated fatty acids (Berge *et al.* 1995), production of FAHs through oxidation of fatty acids by LOX enzymes should take place in the cell lysate even without the addition of exogenous fatty acids. Adding exogenous fatty acids should increase FAH production due to increased substrate availability, but endogenous fatty acids should suffice for supporting LOX

activity in the cell lysate. Therefore LOX activity measured by FAH production in the colorimetric assay was defined as an increase in FAH concentrations in the sample compared to the blank.

Comparative analyses of fresh and frozen samples demonstrated that LOX activity was retained during the freezing process and storing frozen samples at -80°C even for several years significantly altered neither LOX activity nor the production of oxylipins in the sample. Therefore, freezing and storing phytoplankton samples at -80°C is a valid method for collecting field samples for the analysis of oxidative fatty acid metabolism in phytoplankton.

Several considerations need to be taken into account when applying and interpreting the colorimetric assay. First of all, the response of the colorimetric assay was linear only up to a certain concentration of FAH (~90  $\mu$ M). At high FAH concentrations, there seemed to be a saturation of the secondary colorimetric reaction, leading to a loss of linearity. Therefore it is important to have some idea beforehand as to the possible amount of FAHs produced by a sample to adjust the assay accordingly. In general, it is advisable to carry out the colorimetric assay at low sample concentrations to avoid FAH production outside the linear range, also because there seemed to be no compromise of linearity with cell lysate at low concentrations. Even though the limit determined for linearity with a culture of *S. marinoi* was 16 mg wet weight, field phytoplankton samples often showed saturation at lower sample amounts. This was probably due to the fact that field samples were collected from mixed phytoplankton assemblages, thereby not being directly comparable to a pure culture, even though they were dominated by *S. marinoi*. LOX activity in field phytoplankton samples was therefore calculated as FAH production in the more diluted sample (4 mg wet weight) to avoid problems with saturation at higher sample amounts and

a possible underestimation of LOX activity (see 2.2.3). The increased linearity of the polarographic compared to the colorimetric assay was probably due to the fact, that the former assay was based on a direct measurement of LOX activity, whereas the latter relied on a secondary reaction, which apparently went into saturation.

Sodium dodecyl sulphate (SDS) at 0.3% final concentration was used to stop the LOX reaction in the colorimetric assay prior to centrifuging and reading absorbance in the blanks and samples. The lack of an increase in values observed between the blanks and the samples in certain cases was initially attributed to an inability of the detergent to efficiently block LOX enzymes, resulting in an overestimated absorbance reading of blanks. Therefore other ways were examined for stopping the reaction. The use of organic solvents did not prove successful as this led to interferences, possibly due to extraction of chlorophyll by the organic solvent. As expected, boiling was efficient in blocking LOX activity, which also indicated that FAH production was indeed due to enzymatic activity and not dependent on non-enzymatic processes. However, when boiling was used to terminate the reaction prior to reading the absorbance after 20 min, colour formation continued. Apparently, although LOX enzymes were blocked by boiling and no further FAHs were produced, formation of the colour compound was not blocked. This suggested, that also the inability of SDS to block the increase in absorbance over time was not due to an inability of the detergent to block enzyme activity, but due to instability of the colour compound, which continued to be formed even after FAH production was stopped. Indeed it was found with polarographic measurements that 0.3% SDS efficiently blocked LOX activity. In this way it was demonstrated unambiguously, that the continued increase in absorbance after SDS-addition was not due to inability of the detergent to block enzymes, but due to instability of the colour compound. Since this could not be blocked, it was

imperative to read samples at precise time intervals after the initiation of the colorimetric reaction.

Polarographic measurements had the advantage that they were more flexible to changing parameters such as pH. In fact, LOX activity measurements of phytoplankton samples in the oxygraph were carried out in buffer at pH=8.15, because it was assumed that LOX enzymes would encounter this pH upon cell lysis at sea. The colorimetric assay, however, could not be adapted to this pH, because the colorimetric reaction itself was pH-dependent. Via polarographic measurements, it could be shown that the decrease in absorbance with increasing pH in the colorimetric assay was not due to decreasing enzyme activity, but indeed due to a diminishing sensitivity of the secondary colorimetric reaction. This also indicated that not all phytoplankton samples were active at the pH of the colorimetric assay (see Chapter 4). Therefore, if LOX enzymes were not active at the pH of the assay (pH=6.0), as was the case in certain clones of *S. marinoi*, the colorimetric assay underestimated or failed to demonstrate LOX activity, which explains why in some lysates there was no increase from the blank to the sample. Therefore, LOX activity determined with the colorimetric assay could not be calculated as the difference between the sample and the blank in field phytoplankton samples (see 2.2.3). In certain field samples there was neither an increase from the blank to the sample nor an increase when LOX substrate was augmented by adding external EPA to the cell lysate. If LOX enzymes in the particular clone or clones of *S. marinoi* which dominated the bloom in 2005 were not active at pH=6.0, this would explain why the colorimetric assay could not demonstrate LOX activity in these cell lysates, even though samples were producing oxylipins. However, FAH concentrations determined in samples after 20 min without considering blanks, varied between field phytoplankton samples collected on different dates. Since samples were always analyzed in the same way, respecting the same times between sonication and



starting of the assay, the colorimetric assay probably measured the amount of FAHs produced in the sample in the time period between sonication and the start of the colorimetric reaction at the neutral pH of deionised water and were not due to LOX activity during the 20 min of assay duration. This indicates that the colorimetric assay can also be used to dose FAH concentrations in samples. For these reasons, LOX activity in field phytoplankton samples was defined as the FAH concentration measured in the sample after 20 min (see 2.2.3), since FAH concentrations should represent LOX activity in the sample before the start of the colorimetric reaction.

Standard protocol was to suspend phytoplankton pellets in  $\text{H}_2\text{O}_{\text{deionised}}$  (pH=7.0) (d'Ippolito *et al.* 2003). Apparently LOX enzymes were oxidizing fatty acids to FAHs at this pH. However, an improvement for further studies would be to suspend cells at pH=8.15 to allow LOX enzymes to be active at the pH presumably encountered at sea. It is also imperative to closely respect the time period between sonication and the start of the colorimetric assay to allow comparisons between FAH concentrations of different samples. Alternatives for reading blanks should also be taken into consideration, such as suspending samples in  $\text{H}_2\text{O}_{\text{deionised}}$  to correct for absorbance by chlorophyll or other cell lysate compounds or by boiling a part of the sample before sonication to preclude FAH formation in the cell lysate. Also the extraction of samples for quantification of oxylipin production should be carried out with samples suspended at pH=8.15 e. g. in filtered seawater, as the pH influences the amount of oxylipins produced in 30 min.

### 3.5 Conclusions

Both the colorimetric and the polarographic assays were applicable to the determination of lipoxygenase (LOX) activity in phytoplankton lysates. The advantage of the polarographic assay was its flexibility to the pH-value of the buffer employed, since it directly measured the consumption of one of the reactants, oxygen. It therefore exhibited a linear relationship between measured LOX activity and the amount of LOX enzyme. This was not the case with the colorimetric assay because it depended on a secondary colorimetric reaction which went into saturation. The colorimetric assay was also limited by the instability of the colour compound and its dependency on pH=6.0. In general, the pH-value played an important role in determining LOX metabolism because also oxylipin production of the cell lysate seemed to depend on the pH-value of the buffer used for suspending the cells before sonication and extraction. Both assays could be combined to obtain a more complete view of LOX metabolism. Although the colorimetric assay failed to demonstrate activity when the phytoplankton sample was not active at pH=6.0, it could nevertheless be used to show LOX activity in the sample independently of exogenous fatty acids. The polarographic assay, on the other hand, had the advantage that it could demonstrate LOX activity at pH-values other than 6.0. However, it was dependent on the addition of exogenous fatty acids and could thereby only demonstrate part of the LOX activity present in the cell lysate. Freezing samples had no influence on LOX activity measurements and can therefore be employed as a method to collect and store samples until analysis.

## **4 Diversity of Lipoxygenase Metabolism among Different Diatom Species and among Different Clones of the Same Species**

One of the reasons for the often contradictory results obtained for diatom-copepod interactions at sea may be differences in lipoxygenase (LOX) metabolism among different diatom species and even among strains of the same species. This variability in the production of oxygenated fatty acid derived metabolites was investigated further by applying the two assays for LOX activity, colorimetric and polarographic, to three diatom species, *Skeletonema marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis* and to four clones of the same species, *S. marinoi*. Furthermore, all samples were extracted for oxylipin quantification. Differences in LOX activity and oxylipin production were observable among species as well as among clones. Another interesting observation made was that employing different assays did not lead to the same results in that the species or clone more active in one assay was not necessarily more active in another. Therefore the three assays for measuring oxylipin metabolism were not readily interchangeable.

## 4.1 Introduction

Several studies have shown that there are species-specific differences in oxylipin production in diatoms (Fontana *et al.* 2007b) with variations even at the strain level (Pohnert *et al.* 2002; Taylor *et al.* 2009). This variability among strains of the same species can express itself in variable effects on the reproductive success of copepods (Ask *et al.* 2006) and therefore may shed light on contradictory results obtained from field studies. To gain further insight into the diversity of oxylipin metabolism in diatoms, lipoxygenase (LOX) activity assays and oxylipin quantification were carried out on three different diatom species, the well studied PUA-producing *Skeletonema marinoi* and *Thalassiosira rotula* and the non-PUA producing species *Chaetoceros affinis* (Fontana *et al.* 2007b; Koski *et al.* 2008). Differences in oxylipin metabolism were furthermore analyzed among various clones of *S. marinoi*, isolated in four different years during the spring diatom bloom in the Northern Adriatic Sea.

Species and isolates were analyzed for differences in LOX activity both colorimetrically and polarographically, as well as for quantitative and qualitative variations in oxylipin production. The pH-dependency of the LOX reaction was also studied by determining LOX activity in the oxygraph at two different pH-values (pH=6.0 and 8.15). Oxygen consumption at pH=6.0 should correspond to fatty acid hydroperoxide (FAH) production, as determined with the colorimetric assay, because both are due to the oxidation of fatty acids by LOX enzymes (Figure 3-1). Therefore LOX activity determined with the oxygraph at pH=6.0 was compared to LOX activity determined colorimetrically at the same pH.

In this chapter, it was also evaluated whether variations in LOX activity and oxylipin production can explain differential negative effects of different diatom species on copepod

reproductive success. Looking at the literature on the reproductive success of copepods on various diatom diets, it is evident that different diatom species induce different responses. *T. rotula*, for example, had a weaker negative effect than *S. marinoi* on the hatching success of copepod eggs when females fed on these species in the laboratory (d'Ippolito *et al.* 2002b; Ceballos and Ianora 2003). Also *C. affinis* seemed to have a weaker negative effect on copepod reproduction than *S. marinoi* in feeding experiments with copepod females (Fontana *et al.* 2007b). These differences in negative impact on copepod reproductive success may be due to differential oxylin metabolism.

## 4.2 Materials and Methods

Culturing and sampling of *Chaetoceros affinis*, *Thalassiosira rotula*, and the different isolates of *Skeletonema marinoi* were carried out as described in Chapter 3 (3.2.1). Samples were analysed for lipoxygenase (LOX) activity with the colorimetric assay as described in Chapter 2 (2.2.3), and with the polarographic assay at pH=6.0 and pH=8.15 with eicosapentaenoic acid (EPA) as external substrate (0.2 mM) as described in Chapter 3 (3.2.2). Samples were extracted with organic solvents for oxylin quantification as described in Chapter 2 (2.2.4).

Clones of *S. marinoi* were isolated from phytoplankton samples collected during the spring diatom bloom in the Northern Adriatic Sea by Marina Cabrini (Laboratorio di Biologia Marina, Trieste) in 1997 and by Francesco Esposito (SZN, Napoli) in 2003, 2004, and 2005 and kept as batch cultures in the laboratory. To compare different species, mean values for *S. marinoi* were calculated from the three clones isolated in 1997, 2004, and 2005. The isolate of 2003 was not considered in calculations of mean values, because it

differed significantly from the other isolates in that it was the only one to demonstrate LOX activity at pH=6.0 (see 4.3.2).

To evaluate the impact of differential oxylipin production by different diatom species on copepods, variations in cell size among diatom species were also taken into consideration. To this end, oxylipin production was normalized for cell carbon to take into account differential feeding of copepods on phytoplankton cells of varying sizes. For the calculation, literature values of cell carbon content were used because cell carbon measurements of the actual cultures used were not available (Table 4-1).

**Table 4-1: Cellular carbon content [pg C cell<sup>-1</sup>] of *Skeletonema marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*, used for calculation of oxylipin production per milligram carbon. Values were obtained from the literature: a) (Carotenuto *et al.* 2002), b) (Koski *et al.* 2008), c) (Ianora and Poulet 1993).**

Species	Carbon content [pg C cell <sup>-1</sup> ]			Average
<i>Skeletonema marinoi</i>	20.7 <sup>a</sup>			<b>20.7</b>
<i>Thalassiosira rotula</i>	121.9 <sup>a</sup>	267±35 <sup>b</sup>	157±15 <sup>c</sup>	
<i>Chaetoceros affinis</i>	137±16 <sup>b</sup>			

4.3 Results

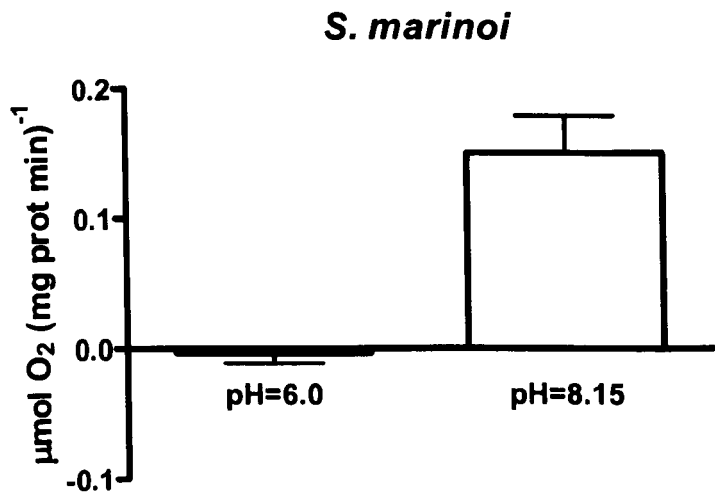
4.3.1 Species Comparison

*Skeletonema marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis* displayed different oxylipin metabolism. This could be shown by the polarographic and the colorimetric

assays, as well as by the production of oxylipins, which demonstrated both qualitative and quantitative differences in the production of fatty acid derived oxygenated metabolites.

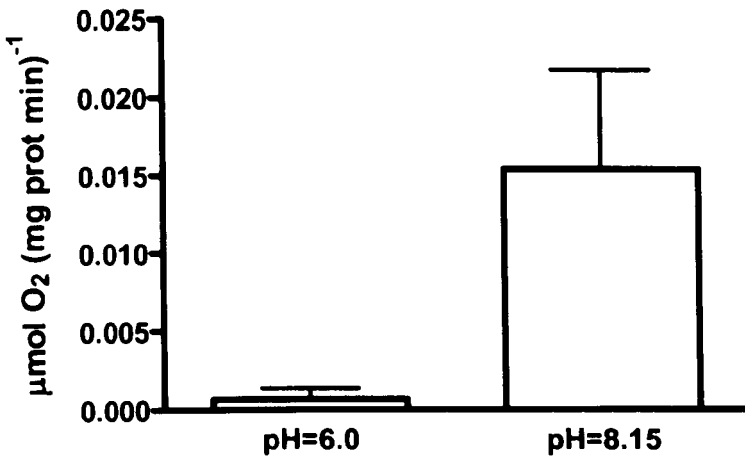
4.3.1.1 Polarographic Assay

Lipoxygenase (LOX) activity in *Skeletonema marinoi* as determined by oxygen consumption rate differed greatly when measured in buffer at pH=6.0 compared to pH=8.15 (paired t-test:  $t=5.87$ ,  $df=8$ ,  $p=0.0004$ ) (Figure 4-1). At pH=8.15, the oxygen consumption rate increased strongly upon addition of eicosapentaenoic acid (EPA), whereas no increase could be observed at pH=6.0.



**Figure 4-1: Mean oxygen consumption rate [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] upon EPA-addition (0.2 mM) (+SEM, n=9) in *Skeletonema marinoi* determined with the polarographic assay at two pH-values (pH=6.0 and pH=8.15).**

### *T. rotula*

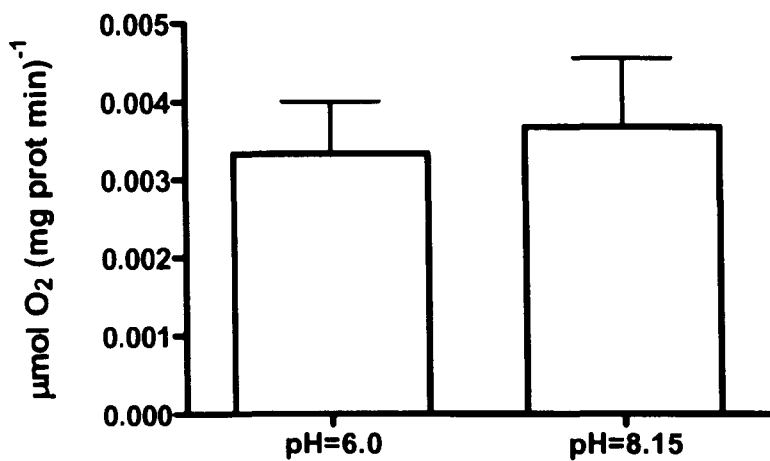


**Figure 4-2:** Mean oxygen consumption rate [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] upon EPA-addition (0.2 mM) (+SEM, n=3) in *Thalassiosira rotula* determined with the polarographic assay at two pH-values (pH=6.0 and pH=8.15).

In *T. rotula*, there were no significant differences in LOX activity measured polarographically at pH=8.15 compared to pH=6.0 (Figure 4-2) (paired t-test:  $p>0.05$ ) In general, the increase in oxygen consumption rate in cell lysate of *T. rotula* upon EPA-addition was about ten times lower than in cell lysate of *S. marinoi* under the same conditions.

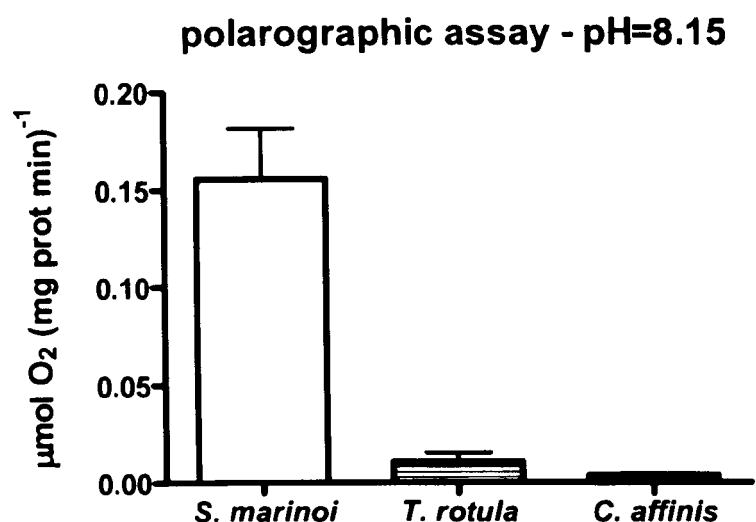


### *C. affinis*



**Figure 4-3: Mean oxygen consumption rate [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] upon EPA-addition (0.2 mM) (+SEM, n=3) in *Chaetoceros affinis* determined with the polarographic assay at two pH-values (pH=6.0 and pH=8.15).**

The increase in oxygen consumption rate upon EPA-addition in cell lysate of *C. affinis* was three times lower than in *T. rotula* and even thirty times lower than in *S. marinoi* (Figure 4-3). There was no difference in activity between the two pH-values tested (paired t-test:  $p > 0.05$ ).



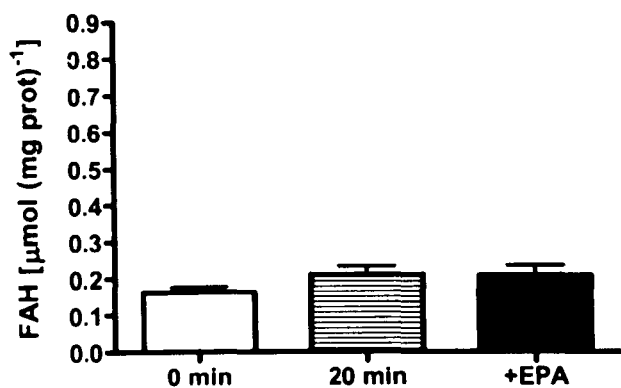
**Figure 4-4: Mean oxygen consumption rate [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] upon EPA-addition (+SEM; *Skeletonema marinoi*: n=12, *Thalassiosira rotula*: n=5, *Chaetoceros affinis*: n=3) determined with the polarographic assay at pH=8.15 in *S. marinoi*, *T. rotula*, and *C. affinis*.**

When polarographic data were compared for the three species at pH=8.15, LOX activity was significantly higher in *S. marinoi* compared to *T. rotula* and *C. affinis* (one-way ANOVA:  $F=10.34$ ,  $r^2=0.55$ ,  $p=0.0012$  and Tukey's post-test:  $p<0.05$ ) (Figure 4-4).

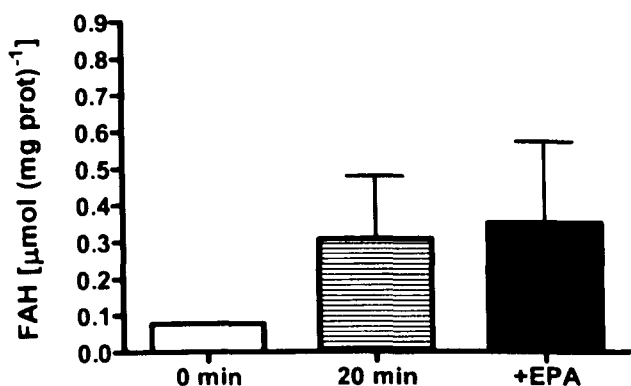
#### 4.3.1.2 Colorimetric Assay

As stated previously (see Chapter 3), except for one isolate, *Skeletonema marinoi* showed almost no activity in the colorimetric assay (Figure 4-5). There was no significant increase of fatty acid hydroperoxide (FAH) concentrations in cell lysate after 20 min compared to blanks and no increase upon addition of exogenous eicosapentaenoic acid (EPA) (one-way ANOVA:  $p>0.05$ ).

***S. marinoi***



***T. rotula***



***C. affinis***

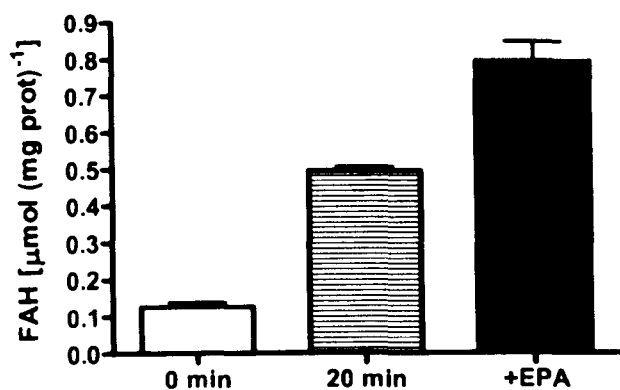
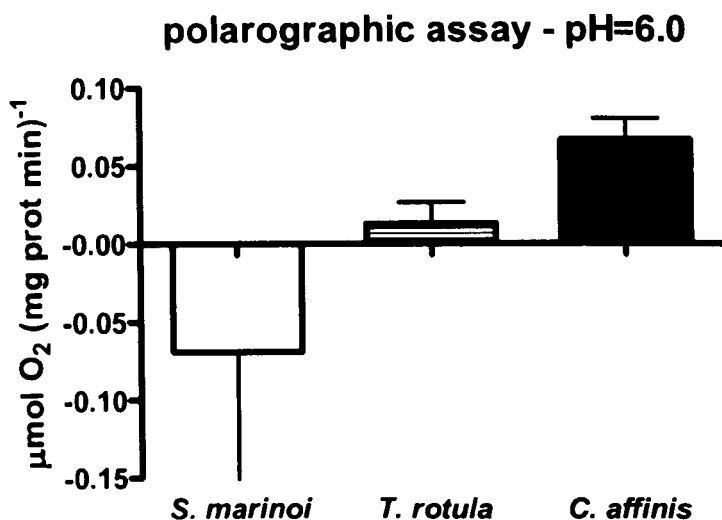


Figure 4-5: Mean concentrations (+SEM, n=3 except *Skeletonema marinoi* n=15) of fatty acid hydroperoxide (FAH) [ $\mu\text{mol (mg prot)}^{-1}$ ] determined with the colorimetric assay in blanks (0 min), in cell lysate of *S. marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis* (20 min) and in cell lysate upon EPA-addition (+EPA).

Also in *Thalassiosira rotula*, there was neither a significant increase of FAH concentrations in the sample compared to the blank, nor upon the addition of EPA to the cell lysate (Figure 4-5) (one-way ANOVA:  $p>0.05$ ). *Chaetoceros affinis*, on the other hand, showed a strong significant increase in FAH concentrations from blanks to samples (one-way ANOVA:  $F=106.9$ ,  $r^2=0.98$ ,  $p=0.0003$  and Tukey's post-test:  $p<0.01$ ). FAH concentrations further increased when samples were incubated with exogenous EPA ( $p<0.01$ ) (Figure 4-5).

With the colorimetric assay, *C. affinis* was the only species to demonstrate significant production of LOX products both with and without EPA-addition. Also oxygen consumption rate upon EPA-addition at pH=6.0 was significantly higher in *C. affinis* compared to *T. rotula* (t-test:  $t=2.88$ ,  $df=4$ ,  $p=0.045$ ), whereas addition of EPA to *S. marinoi* cell lysate led to a decrease in oxygen consumption rate (Figure 4-6). This pattern was opposite to the pattern observed at pH=8.15, where *S. marinoi* lysate had demonstrated significantly higher oxygen consumption rate upon EPA-addition than *C. affinis* lysate (Figure 4-4).



**Figure 4-6: Mean oxygen consumption rate [nmol O<sub>2</sub> (mg prot min)<sup>-1</sup>] upon EPA-addition (+SEM; n=3, except *Skeletonema marinoi*: n=8) determined with the polarographic assay at pH=6.0 in *S. marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*.**

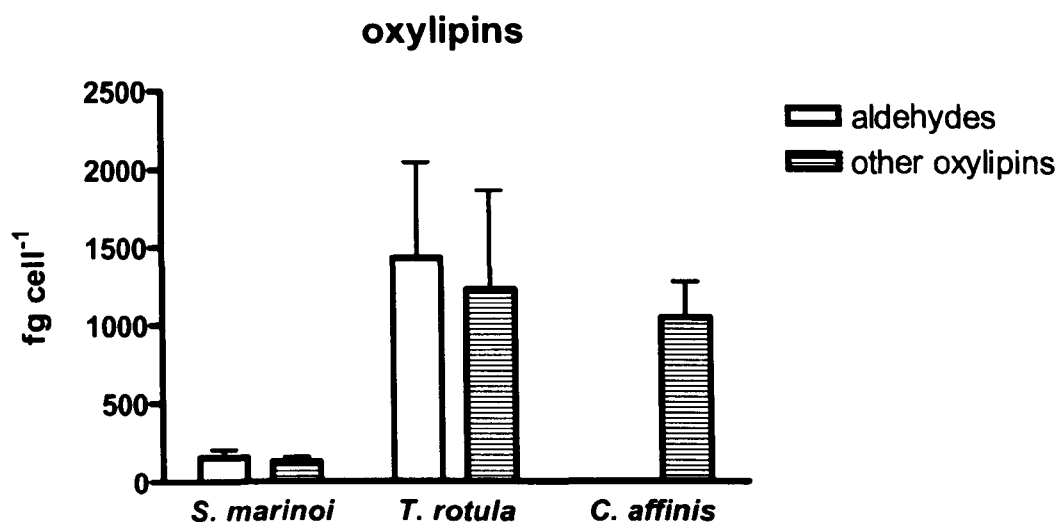
#### 4.3.1.3 Oxylin Production

*Skeletonema marinoi* produced the aldehydes heptadienal, octadienal, and octatrienal, in addition to hydroxy-acids and epoxyalcohols deriving from C<sub>16</sub>- and C<sub>20</sub>-fatty acids (Table 4-2). In accordance with the literature, no decatrilal could be found in extracts of this species (d'Ippolito *et al.* 2002b). *Thalassiosira rotula* on the other hand produced the former three aldehydes in addition to decatrilal and hydroxy-acids and epoxyalcohols from C<sub>16</sub>- and C<sub>20</sub>-fatty acids also concordant with the literature (d'Ippolito *et al.* 2005; Fontana *et al.* 2007a). As described in Fontana *et al.* (2007b), *Chaetoceros affinis* produced aldehydes, epoxyalcohols, and hydroxy-acids derived from eicosapentaenoic acid (C<sub>20</sub>) only (Table 4-2). Representative chromatograms of GC- and LC-MS analyses of all species can be found in Appendix 3.

**Table 4-2: Oxylipins, including the aldehydes heptadienal, octadienal, octatrienal, and decatrienal and the epoxyalcohols (EPOX) and hydroxy-acids (OH) produced from hexadecatrienoic acid (C<sub>16:3</sub>) and hexadecatetraenoic acid (C<sub>16:4</sub>), as well as eicosapentaenoic acid (C<sub>20</sub>), produced by *Skeletonema marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*.**

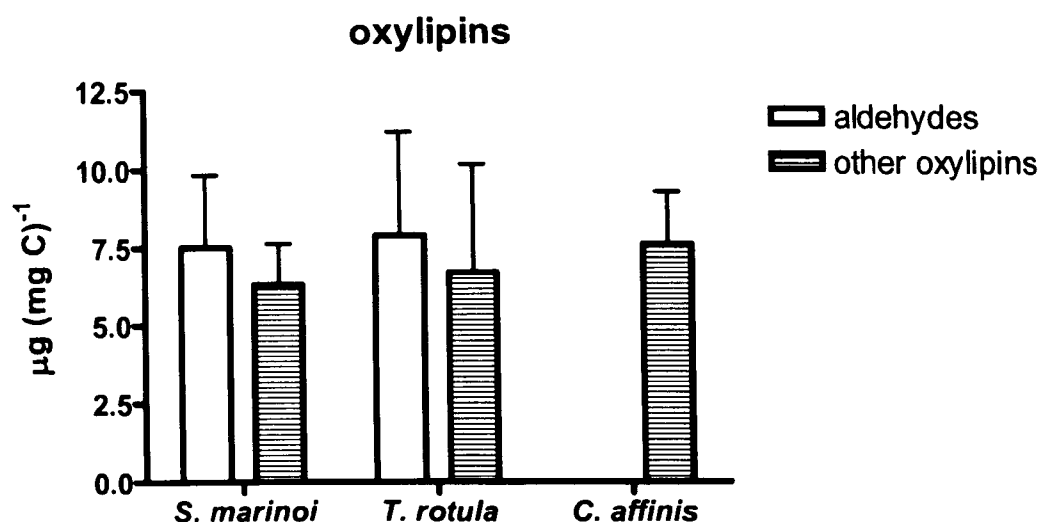
	<i>Skeletonema marinoi</i>	<i>Thalassiosira rotula</i>	<i>Chaetoceros affinis</i>
<b>Aldehydes</b>			
<b>Heptadienal</b>	X	X	
<b>Octadienal</b>	X	X	
<b>Octatrienal</b>	X	X	
<b>Decatrienal</b>		X	
<b>Other oxylipins</b>			
<b>C<sub>16:3</sub>-OH</b>	X	X	
<b>C<sub>16:3</sub>-EPOX</b>	X	X	
<b>C<sub>16:4</sub>-OH</b>	X	X	
<b>C<sub>16:4</sub>-EPOX</b>	X	X	
<b>C<sub>20</sub>-OH</b>	X	X	X
<b>C<sub>20</sub>-EPOX</b>	X	X	X

Highest cellular production of both aldehydes and other oxylipins was observed in *T. rotula* (1430±611 fg cell<sup>-1</sup> and 1226±634 fg cell<sup>-1</sup>) (Figure 4-7), even though variability among replicates of this species was very high. Aldehyde production in *T. rotula* was significantly higher than in *S. marinoi* (t-test: *t*=4.46, *df*=13, *p*=0.0006). Production of oxylipins other than aldehydes in *T. rotula* was also significantly higher than in *S. marinoi* (one-way ANOVA: *F*=10.8, *r*<sup>2</sup>=0.59, *p*=0.0013 and Tukey’s post-test: *p*<0.01) but not significantly different from *C. affinis* (*p*>0.05).



**Figure 4-7: Mean production (+SEM; n=3 except *Skeletonema marinoi*: n=12) of aldehydes and other oxylipins normalized for cells [fg cell<sup>-1</sup>] in *S. marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*.**

Production of oxylipins other than aldehydes was significantly higher in *C. affinis* than *S. marinoi* ( $p < 0.01$ ). However, since *C. affinis* did not produce aldehydes, oxylipin production did not differ significantly between *S. marinoi* and *C. affinis* (one-way ANOVA:  $F=8.56$ ,  $r^2=0.40$ ,  $p=0.001$  and Tukey's post-test:  $p > 0.05$ ).



**Figure 4-8: Mean production (+SEM; n=3 except *Skeletonema marinoi*: n=12) of aldehydes and other oxylipins normalized for cell carbon [ $\mu\text{g (mg C)}^{-1}$ ] in *S. marinoi*, *Thalassiosira rotula*, and *Chaetoceros affinis*.**

When aldehyde production was normalized for cell carbon, there were no differences between *S. marinoi* and *T. rotula* (t-test:  $p>0.05$ ) (Figure 4-8). Also production of oxylipins other than aldehydes normalized for cell carbon did not differ between species (one-way ANOVA:  $p>0.05$ ). When aldehydes and oxylipins other than aldehydes were considered together, there were no differences in oxylipin production among the three species.

#### **4.3.2 Comparison of Different Clones of *Skeletonema marinoi***

Also the four clones of *Skeletonema marinoi*, isolated in different years during the diatom bloom, demonstrated differences in oxylipin metabolism. These differences could be demonstrated by the colorimetric and polarographic assays, as well as by oxylipin analysis.



4.3.2.1 Colorimetric Assay

There were no significant increases in fatty acid hydroperoxide (FAH) concentrations from the blank (0 min) to the sample (20 min) or upon EPA-addition to the sample (+EPA) in the *S. marinoi* clones isolated in 1997, 2004, and 2005 (one-way ANOVAs,  $p>0.05$ ) (Figure 4-9).

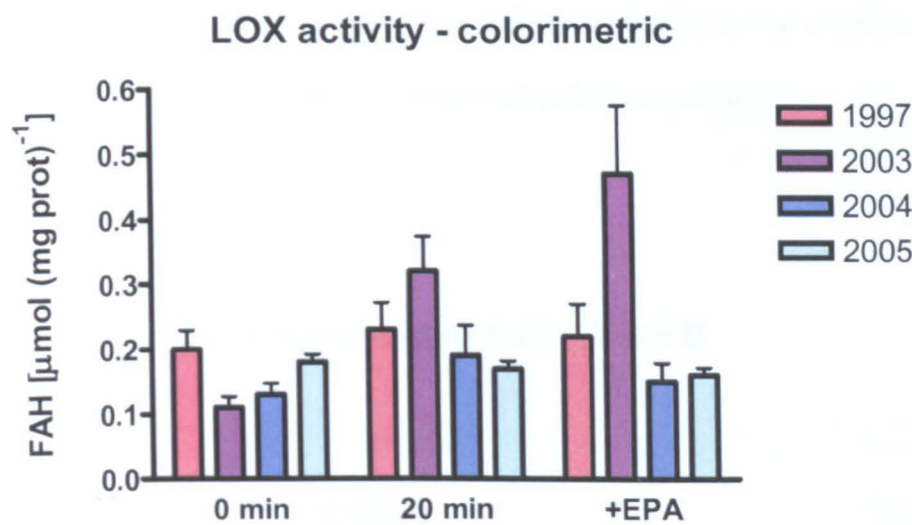


Figure 4-9: Mean concentrations (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) of fatty acid hydroperoxide (FAH) [ $\mu\text{mol (mg prot)}^{-1}$ ] determined with the colorimetric assay in blanks (0 min), in cell lysate (20 min) and in cell lysate upon EPA-addition (+EPA) of the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005.

One isolate, however, differed from this general pattern. This was the clone isolated during the bloom in 2003, which showed strong activity in the colorimetric assay with a significant difference in FAH concentrations between the blank (0 min) and the sample upon EPA-addition (+EPA) (one-way ANOVA:  $F=5.49$ ,  $r^2=0.34$ ,  $p=0.012$  and Tukey's post-test:  $p<0.01$ ) (Figure 4-9).

4.3.2.2 Polarographic Assay

Lipoxygenase (LOX) activity, as determined with the colorimetric assay for the various clones, was compared to LOX activity measured in the oxygraph at the same pH (Figure 4-10). Meaningful statistical analyses could not be carried out because of the low number of replicates for the clone from 2005 (n=2). However, the only isolate to demonstrate LOX activity in the colorimetric assay (2003) seemed to exhibit strongest LOX activity at pH=6.0 in the polarographic assay (Figures 4-9 and 4-10). Also the slight decrease in LOX activity upon EPA-addition indicated in the colorimetric assay with the isolates from 2004 and 2005 was observed polarographically.

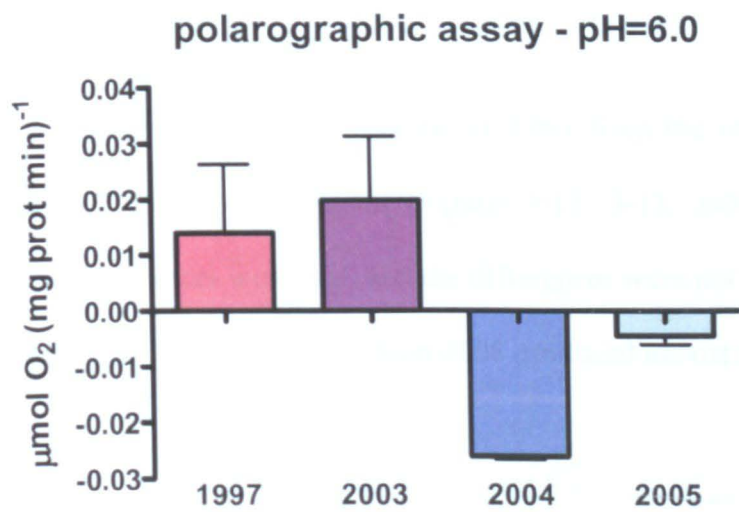


Figure 4-10: Mean oxygen consumption rate [nmol O<sub>2</sub> (mg prot min)<sup>-1</sup>] upon EPA-addition (+SEM; 1997: n=4, 2003: n=5, 2004: n=3, 2005, n=2) determined with the polarographic assay at pH=6.0 in the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005.

When LOX activity was measured at pH=8.15 this pattern changed (Figure 4-11). At pH=8.15 there were no significant differences in LOX activity between clones with EPA as external substrate (one-way ANOVA:  $p>0.05$ ).

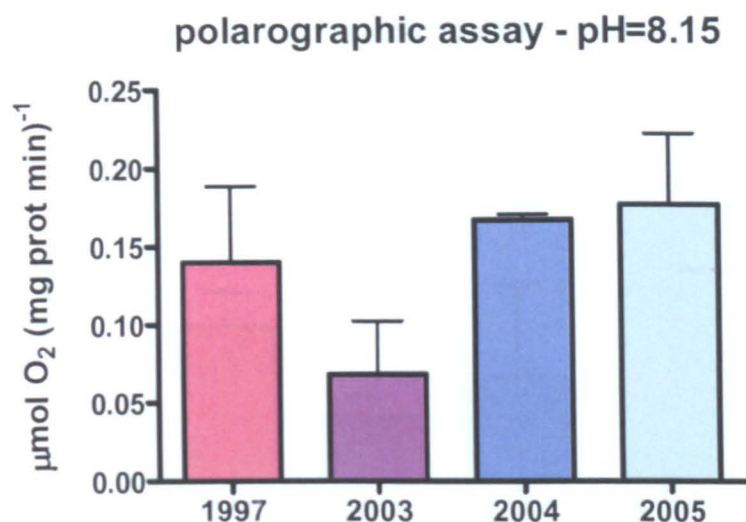


Figure 4-11: Mean oxygen consumption rate [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] upon EPA-addition (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) determined with the polarographic assay at pH=8.15 in the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005.

#### 4.3.2.3 Oxylipin Production

The clone isolated in 2005 seemed to differ from the other clones in the production of aldehydes and other oxylipins (Figures 4-12, 4-13, and 4-14), even though variability between replicates were high and the differences were not statistically significant (one-way ANOVA:  $p>0.05$ ). The clone from 2005 produced almost no aldehydes (Figure 4-12).

## aldehydes

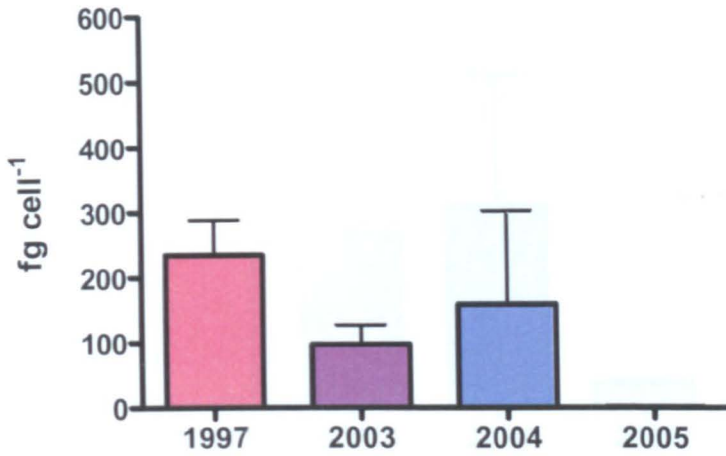


Figure 4-12: Mean aldehyde production (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) normalized for cells [fg cell<sup>-1</sup>] in the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005.

## production of other oxylipins

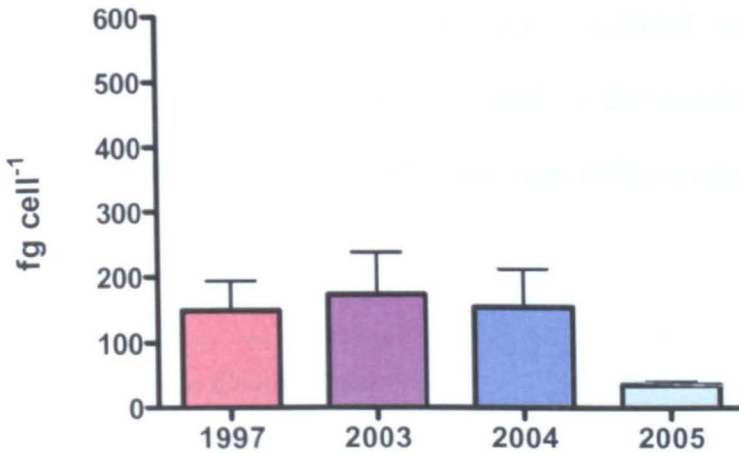


Figure 4-13: Mean production (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) of oxylipins other than aldehydes normalized for cells [fg cell<sup>-1</sup>] in the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005.

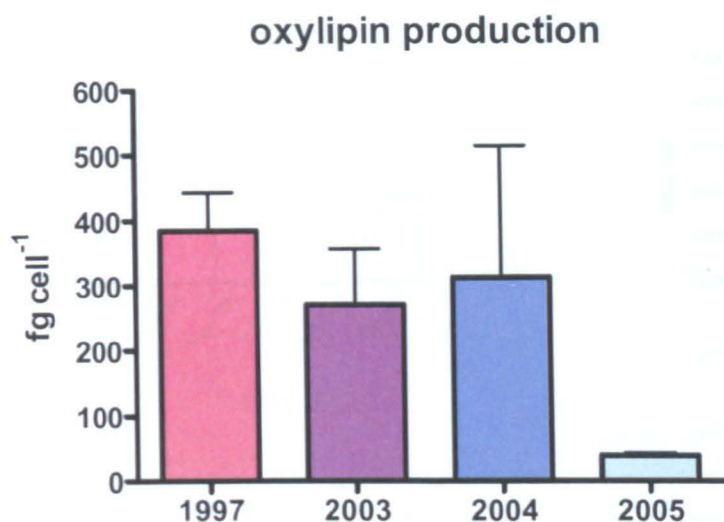


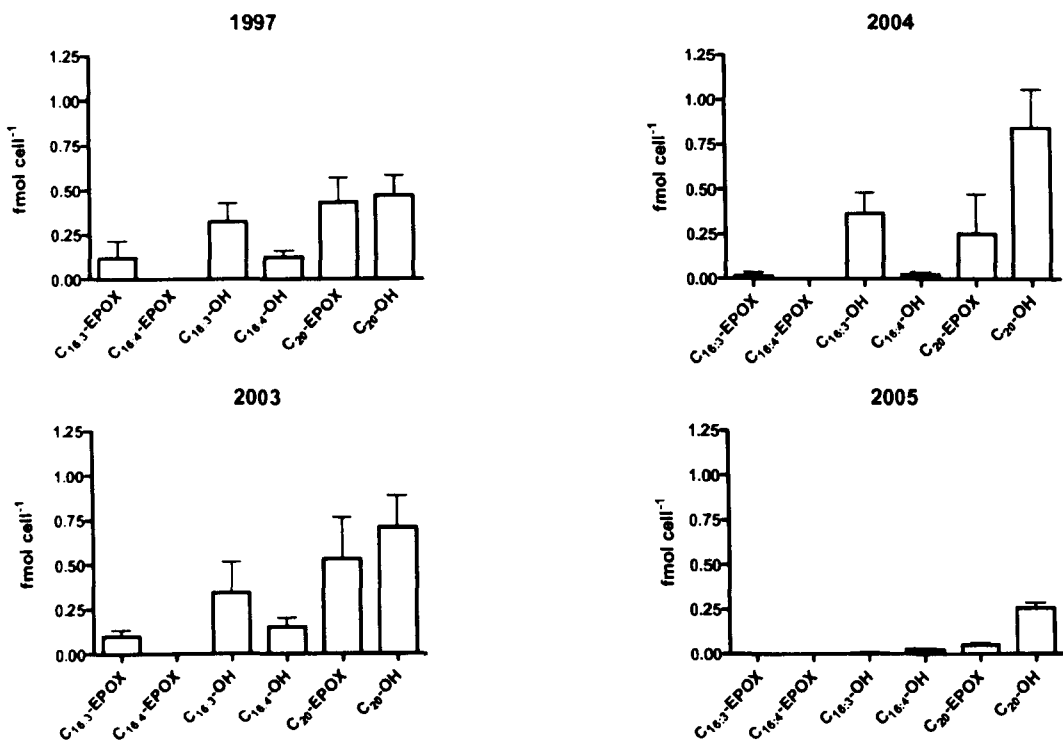
Figure 4-14: Mean oxylipin production (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) normalized for cells [fg cell<sup>-1</sup>] in the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005.

Also the production of oxylipins other than aldehydes was low in the isolate from 2005 at  $42 \pm 7$  fg cell<sup>-1</sup> (Figure 4-13). Aldehyde production and the production of other oxylipins were in the same size range and were considered together as oxylipin production (Figure 4-14). This appeared to be lowest in the isolate from 2005 at  $44 \pm 7$  fg cell<sup>-1</sup>, whereas the other three clones did not seem to differ in oxylipin production.

#### 4.3.2.4 Oxylipin Composition

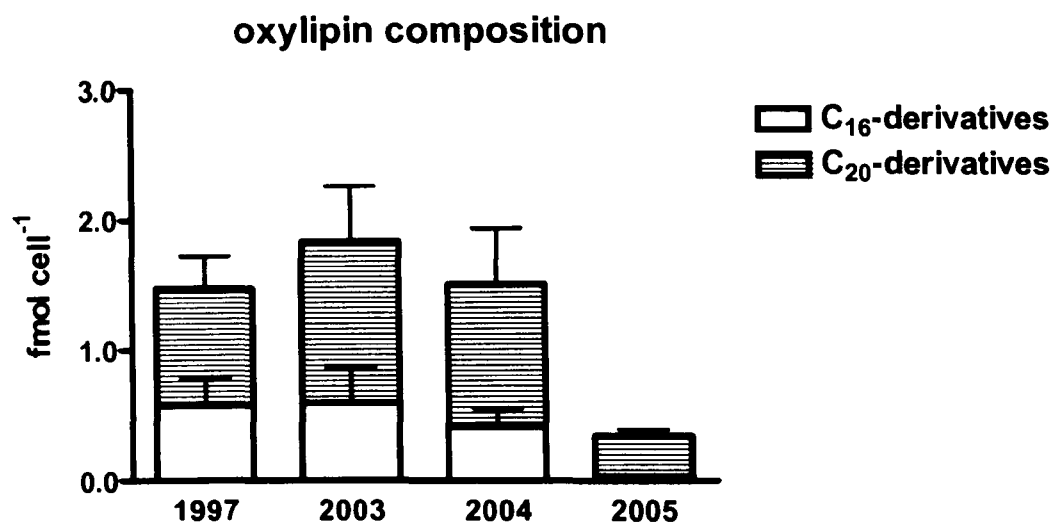
There were significant differences in oxylipin composition between clones (two-way ANOVA: interaction:  $p > 0.05$ , clone:  $p = 0.0023$ , oxylipin:  $p < 0.0001$ ). Composition of the oxylipin production by the two isolates from 1997 and 2003 did not differ (Bonferroni post-test:  $p > 0.05$ ) (Figure 4-15).





**Figure 4-15: Mean oxylipin composition (+SEM; 1997; n=6, 2003: n=9, 2004 and 2005: n=3) normalized for cells [fmol cell<sup>-1</sup>] in the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005 (EPOX and OH: epoxyalcohols and hydroxy-acids derived from C<sub>16:3</sub>-, C<sub>16:4</sub>-, and C<sub>20</sub>-fatty acids, respectively).**

The isolate from 1997 also did not differ significantly from the two clones isolated in 2004 and 2005 in terms of oxylipin production ( $p>0.05$ ). The significant difference in oxylipin composition between isolates was the production of the C<sub>20</sub>-epoxyalcohol in 2003 compared to 2004 and 2005 ( $p<0.05$ ). All in all, the same oxylipin metabolites were produced by the various clones, although there was some indication as to differential production of individual oxylipin compounds. Initial visual analysis showed that the spectra of the products formed by the clones isolated in 1997 and 2003 seemed the same, whereas production in 2004 and 2005 seemed to be shifted more towards the C<sub>20</sub>-hydroxy-acid (Figure 4-15).



**Figure 4-16:** Mean contributions (+SEM; 1997; n=6, 2003: n=9, 2004 and 2005: n=3) of C<sub>16</sub>- and C<sub>20</sub>-derivatives to oxylipin composition [fmol cell<sup>-1</sup>] in the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005.

The contribution of C<sub>20</sub>-derivatives to the production of oxylipins other than aldehydes was significantly higher than the contribution of C<sub>16</sub>-derivatives in the isolate from 2005 (paired t-test:  $t=6.30$ ,  $df=2$ ,  $p=0.024$ ) (Figure 4-16). In the other isolates, the contribution of C<sub>16</sub>-fatty acids did not differ significantly from the contribution of C<sub>20</sub>-fatty acids to the production of oxylipins other than aldehydes. However, there seemed to be a ratio of about 1:2 (C<sub>16</sub>:C<sub>20</sub>).

#### 4.3.2.5 Other Parameters

Protein content in the cell lysates did not differ significantly between the different clones of *Skeletonema marinoi* (Figure 4-17) (ANOVA:  $p>0.05$ ). Also growth rates were not significantly different (ANOVA:  $p>0.05$ ). All clones grew at about the same rate of  $0.76 \pm 0.04 \text{ d}^{-1}$  (Figure 4-18).

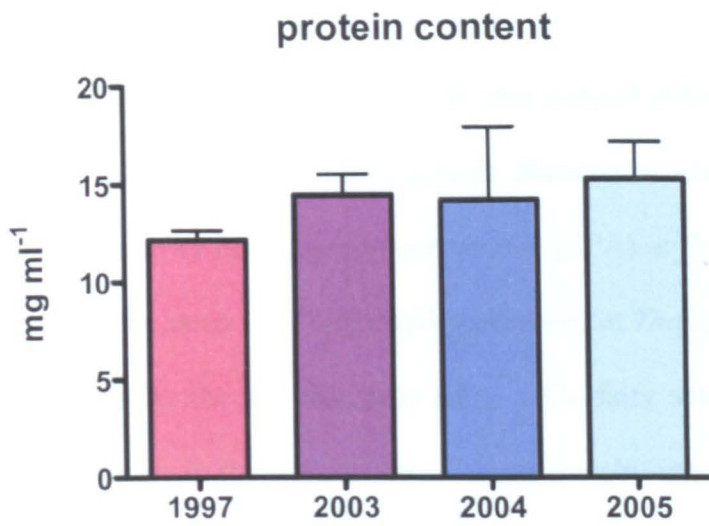


Figure 4-17: Mean protein concentration (+SEM; 1997: n=6, 2003: n=9, 2004 and 2005: n=3) [mg prot ml<sup>-1</sup>] in cell lysate of the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005.

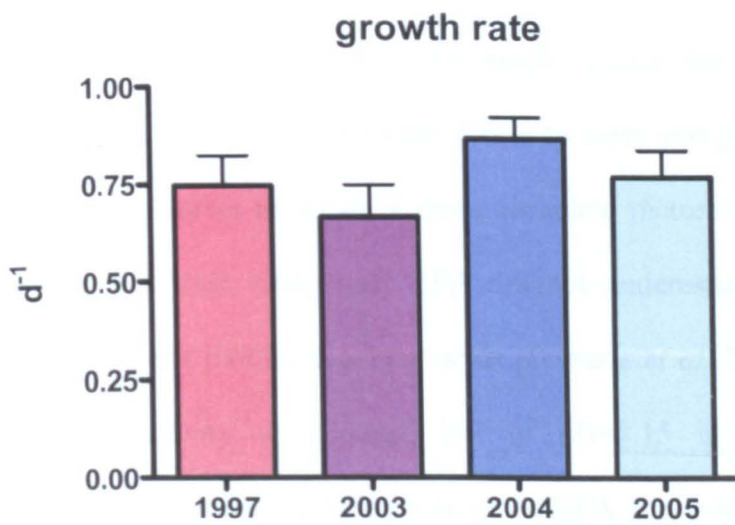


Figure 4-18: Mean growth rate (+SEM, 1997: n=3, 2003: n=3, 2004: n=6, 2005: n=4) [d<sup>-1</sup>] of the *Skeletonema marinoi* clones isolated in 1997, 2003, 2004, and 2005.



## 4.4 Discussion

The polarographic measurements indicated distinct differences in the oxidative metabolism of fatty acids in different diatom species. *Skeletonema marinoi* apparently had the strongest oxidizing activity on eicosapentaenoic acid (EPA) at the pH of seawater (pH=8.15) in the polarographic assay. The low activity observed in *Thalassiosira rotula* may, however, have been biased by the fact that only EPA, a C<sub>20</sub>-fatty acid was used to detect lipoxygenase (LOX) activity in the polarographic assay, thereby ignoring LOX metabolism of C<sub>16</sub>-fatty acids in the cell lysate. Oxylipin composition of *S. marinoi* demonstrated a relationship of oxylipins deriving from C<sub>20</sub>-fatty acids to oxylipins deriving from C<sub>16</sub>-fatty acids of about 2:1, suggesting that using only EPA in the oxygraph underestimated LOX activity in *S. marinoi*. In *T. rotula*, however, metabolism of C<sub>16</sub>-fatty acids is predominant (A. Fontana, pers. comm.). Therefore, the underestimation of LOX activity in the oxygraph by adding just EPA was probably much greater for *T. rotula* cell lysates than for *S. marinoi*. It would be best in the future to carry out polarographic measurements also with a C<sub>16</sub>-substrate to obtain a more complete picture of LOX activity. In the case of *Chaetoceros affinis*, using only EPA did not underestimate LOX activity because this species does not oxidize C<sub>16</sub>-fatty acids (Fontana *et al.* 2007b). Therefore it would seem that LOX activity was indeed low at pH=8.15 in *C. affinis*. Whereas *S. marinoi* demonstrated stronger oxidizing activity on EPA at pH=8.15 than pH=6.0, LOX activity in *C. affinis* seemed independent of pH. In fact, at pH=6.0, *C. affinis* was the species with strongest LOX activity, demonstrable both by the polarographic assay carried out at this pH and by the colorimetric assay.

LOX activity measured at the pH of seawater (pH=8.15) should theoretically demonstrate LOX activity when cells break open in seawater and therefore should be most closely

correlated to the effect on copepods. Stronger LOX metabolism in *S. marinoi* at pH=8.15 would indeed be in accordance with a stronger negative effect observed for *S. marinoi* on copepod reproductive success compared to *T. rotula* and *C. affinis* (d'Ippolito *et al.* 2002b; Ceballos and Ianora 2003; Fontana *et al.* 2007b). This suggests that measuring LOX activity in the oxygraph at pH=8.15 may indeed be a good method for determining the potential damage on copepod reproduction by diatom oxylipin metabolism. Oxylipin production, on the other hand, did not correlate to the detrimental impact of different diatom species on copepod reproduction. Cellular oxylipin production was actually lowest in *S. marinoi* and when oxylipin production was normalized for cell carbon, which is probably more relevant for copepod ingestion, there were no significant differences observable between species. However, *C. affinis* did not produce aldehydes, which have shown to have a stronger effect on copepod reproduction than other oxylipins (Fontana *et al.* 2007b). The weaker negative effect of *C. affinis* on copepod reproduction compared to *S. marinoi*, as described in the literature (Fontana *et al.* 2007b), may therefore be due to a lack of the more toxic metabolites, aldehydes, in the former species.

Diversity in LOX metabolism was also detectable among different isolates of *S. marinoi*. Protein content and growth rate did not differ among isolates, indicating that there were no major metabolic differences among the clones isolated in different years. However, there were differences in oxidative fatty acid metabolism. Apart from apparent quantitative differences in oxylipin production, clones also differed in the relative contributions of different oxylipin compounds. This suggests that oxylipin metabolism may be a very sensitive method to distinguish among different clones of the same species.

It is interesting to note that the isolate from 2003 was the only isolate to show significant LOX activity by the production of fatty acid hydroperoxides (FAHs) at pH=6.0 in the

colorimetric assay. This isolate had been collected in a year in which the diatom bloom had had a very strong negative effect on copepod hatching success (see Chapter 2). Importantly, the production of FAHs in different clones, as determined by the colorimetric assay, did not correspond to the production of end-metabolites, such as aldehydes and other oxylipins. The only clone to demonstrate LOX activity in the colorimetric assay (2003) did not differ in oxylipin production from two of the other isolates. Also when LOX activity was measured at pH=8.15 in the polarographic assay, there was no correlation between LOX activity and oxylipin production. All isolates had similar LOX activity at pH=8.15, even though the clone isolated in 2005 seemed to produce lower amounts of oxylipins compared to the other three isolates.

To conclude, differences in oxylipin metabolism exist among different diatom species and even among clones of the same species isolated in different years. Variability between strains has also been shown by Taylor *et al.* (2009) for aldehyde production, which varied among different strains of *S. marinoi* isolated at sea during different time periods. These variations in LOX activity among isolates from different years may explain some of the variable results observed in the field, thereby adding isolate-dependent variability as another important facet to the increasingly complex picture of oxylipin metabolism in diatoms.

## 4.5 Conclusions

To conclude, differences in lipoxygenase (LOX) metabolism were detectable in different diatom species, as well as in clones of the same species isolated in different years. Whereas *Skeletonema marinoi* and *Thalassiosira rotula* failed to show activity with the colorimetric assay, *Chaetoceros affinis* showed strong production of LOX products, fatty acid hydroperoxides (FAHs), at pH=6.0. *S. marinoi* on the other hand demonstrated strong LOX metabolism on eicosapentaenoic acid (EPA) at pH=8.15 in the polarographic assay. Nonetheless, oxylipin production per cell was low in *S. marinoi*. However, oxylipin production normalized per cell carbon was the same among species. As far as the different isolates of *S. marinoi* collected during the winter diatom bloom in the Northern Adriatic Sea in the years 1997, 2003, 2004, and 2005 were concerned, the *S. marinoi* clone isolated in 2003 was the only one to exhibit LOX activity via the production of FAHs at pH=6.0 in the colorimetric assay. However, at pH=8.15 all clones demonstrated similar activity on EPA in the polarographic assay. Production of aldehydes, as well as oxylipins other than aldehydes was low in the isolate from 2005, whereas the other isolates did not seem to differ in oxylipin production. Oxylipin composition also seemed to be shifted more towards EPA-derivatives in the 2005 clone. Lastly, there was no correspondence between neither of the two assays for LOX activity and the production of oxylipins as end-metabolites. This indicates that there is still something missing in our knowledge of diatom oxylipin metabolism to be able to correlate LOX activity to oxylipin production.

## **5 Effect of Phosphorus-limitation on Lipoxygenase Activity of *Skeletonema marinoi* and its Effects on the Reproductive Success of *Calanus helgolandicus***

Previous studies have demonstrated that nutrient stress induces an increase in aldehyde production in the diatom *Skeletonema marinoi*. Therefore, the hypothesis was put forth that these nutrient-stressed diatoms should have a stronger detrimental impact on copepod reproductive success. To test this hypothesis, *S. marinoi* was grown under phosphorus (P)-limitation in a chemostat. The outflow culture, consisting of P-limited algae, was then used to feed females of *Calanus helgolandicus*. Females were followed for faecal pellet production, egg production, hatching success and the occurrence of abnormal nauplii during the two-week incubation experiments and these parameters were compared to those obtained from females feeding on nutrient-replete *S. marinoi*. P-limited and P-replete diatoms were analyzed for lipoxygenase (LOX) activity and oxylin production. Mass spectrometric analyses showed that P-stress did not increase oxylin production in the present experiment. LOX activity assays actually indicated increased LOX metabolism in P-replete cultures, which was contradictory to expectations. Correspondingly, there were no clear effects of the different treatments on copepod reproduction. Hatching success of nauplii actually seemed less effected when females fed on P-limited alga, which was unexpected. However, feeding on P-limited alga increased the percentage of abnormal nauplii. In general, both treatments strongly reduced hatching success of nauplii.

## 5.1 Introduction

*Skeletonema marinoi* negatively impacts the reproductive success of calanoid copepods, including *Calanus helgolandicus*, through the production of fatty acid derived oxygenated metabolites such as aldehydes and other oxylipins (d'Ippolito *et al.* 2002b; Fontana *et al.* 2007b). Recently, it has been reported that nutrient-limited cultures of this diatom species produce greater amounts of aldehydes (PUAs) than control nutrient-replete cultures (Ribalet *et al.* 2007b; Ribalet *et al.* 2009). Apparently, nutrient-limitation induces a stress in the algal cells leading to increased lipoxygenase (LOX) activity with a resulting stronger production of oxylipins. The hypothesis for the following experiments was that these nutrient-limited cultures would then have a stronger deleterious effect on copepod reproduction due to the increased production of fatty acid derived oxygenated metabolites.

Nutrient-limited *S. marinoi* was obtained by continuous culturing of cells in a chemostat (Ribalet *et al.* 2007b; Ribalet *et al.* 2009). Basically, a chemostat allows culturing of bacteria or phytoplankton under constant nutrient deficiency (Novick and Szilard 1950). This is achieved by growing cultures on a culture medium limited in the nutrient of interest under a constant dilution rate. The culture medium is supplied at a fixed rate to a thoroughly mixed growth chamber of fixed volume and the culture medium of the growth chamber together with the culture growing therein removed at the same rate (Caperon 1968). Mixing in the present experimental set-up was guaranteed by magnetic stirring and bubbling of the culture. Upon reaching a steady state, cell growth balances dilution rate and cell concentrations will remain constant. In this state, cells will grow until their growth requirements for the limiting nutrient match the supply of that nutrient (Caperon 1968). If the dilution rate in the chemostat is less than the maximum growth rate, cells will be limited in the nutrient of interest (Thomas and Dodson 1972).

A chemostat set-up was used to culture *S. marinoi* under phosphorus (P)-limiting conditions and the chemostat outflow was used to feed *C. helgolandicus* females. Chemostat samples of *S. marinoi* were analyzed for LOX activity and for oxylin production. Daily faecal pellet production rates, egg production rates, hatching success, and occurrence of abnormal nauplii of *C. helgolandicus* were compared between females fed P-limited *S. marinoi* and females fed control P-replete cultures.

## 5.2 Materials and Methods

### 5.2.1 Experimental Set-up

Chemostats consisted of graduated polycarbonate bottles designed for growing maximum one litre of culture (Figure 5-1). Bottles were closed by a screw-top with an integrated magnetic stirring device and had two side openings which could be sealed air-tight by silicon stoppers. One of the openings allowed for inflow of the culture medium through a silicon tube connected on the other end to a 10-litre bottle containing the culture medium. Culture medium was prepared from natural oligotrophic filtered seawater following the usual protocol for f/2 medium according to Guillard (Guillard 1975), except that the concentration of phosphate was decreased from 36  $\mu\text{M}$  to 11  $\mu\text{M}$  (P-limited medium). The same opening used for medium inflow also accommodated a glass tube for aeration of the culture through bubbling of sterile (0.22- $\mu\text{m}$  filtered) ambient air. Furthermore a silicon tube was passed through this opening to the bottom of the chemostat for culture sample removal via a sterile syringe. The other opening allowed for removal of culture (outflow) via a glass tube connected to a silicon tube which ended in the outflow collecting chamber. This consisted of a two-litre polycarbonate bottle with a second silicon tube allowing for

aeration by sterile (0.22- $\mu\text{m}$  filtered) ambient air to avoid sedimentation of the outflow culture. The silicon tubes for medium inflow and culture outflow were passed through the same channel of a peristaltic pump to guarantee the same flow rate for inflowing medium and effluent culture.



**Figure 5-1:** On the left, the entire chemostat set-up in the culture chamber with the culture medium bottle on the top, the chemostat bottle on the middle, and the outflow bottle on the bottom shelf. On the right, a close-up of the chemostat bottle with inflowing medium, effluent culture and bubbling. Photos are courtesy of François Ribalet.

The entire set-up, apart from the culture medium bottle which was autoclaved separately, was autoclaved together (20 min at 121°C), and everything assembled under the sterile hood. Chemostats were set up on a magnetic stirrer in a climate chamber at 20°C on a 12h:12h light:dark cycle (100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) (Figure 5-1).

The axenic strain of *Skeletonema marinoi* CCMP 2092 (recently separated from *S. costatum* (Sarno *et al.* 2005)) isolated from the Northern Adriatic Sea was used for



inoculation of the chemostat. Axenity was established by inoculating 1 ml of the culture in 0.1% peptone agar in culture medium and incubating in the dark for several days, controlling daily for turbidity as indicative of bacterial growth. The same method was used at regular intervals to control axenity in the chemostat during the course of the experiment. To obtain phosphorus (P)-limited cultures, *S. marinoi* was grown in the chemostat on P-limited medium at a dilution rate corresponding to ~30% of maximum growth rate ( $\mu_{\max}$ ). For control P-replete cultures, the dilution rate was raised to ~90% of  $\mu_{\max}$ . Maximum growth rate ( $\mu_{\max}$ ) was determined directly in the chemostat in triplicate by enumerating daily cell concentrations during exponential growth before starting the chemostat. To obtain a nutrient-limited culture or to switch the culture from a nutrient-limited to a nutrient-replete status, six generations of phytoplankton cells were allowed to pass before starting the incubation experiments with copepods (Ribalet *et al.* 2007b). At 30%  $\mu_{\max}$  for P-limited cultures this time period corresponded to 14 days, whereas 3 days were sufficient for acclimating cells when switching from P-limited to P-replete cultures at 90%  $\mu_{\max}$ .

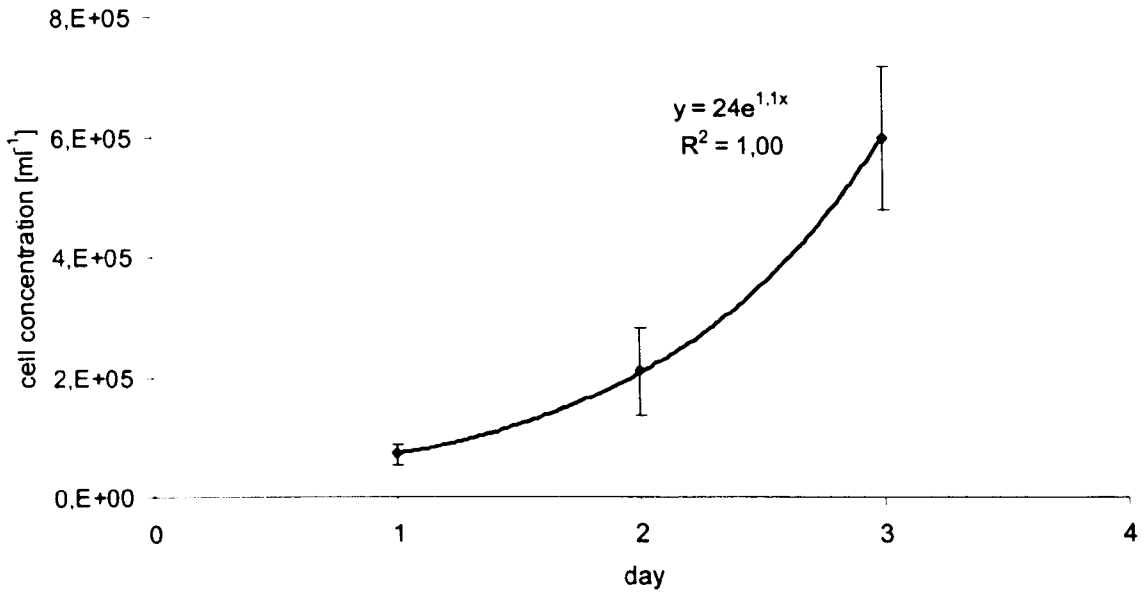
The maximum growth rate ( $\mu_{\max}$ ) for *S. marinoi* was calculated from the following formula:

$$\mu_{\max} = \ln (N_{d+x}/N_d) / ((d+x)-d)$$

$N_{d+x}$  – cell concentration on day  $d+x$

$N_d$  – cell concentration on day  $d$

Mean  $\mu_{\max}$  for *S. marinoi* determined in the chemostat was  $1.1 \pm 0.2 \text{ d}^{-1}$  (Figure 5-2). Therefore, cell numbers were doubling every 16 hours. Accordingly, at 90%  $\mu_{\max}$  half of the total volume ( $V_{\text{tot}}=1000 \text{ ml}$ ) needed to be displaced in 18 hours. This necessitated a flow rate of  $0.47 \text{ ml min}^{-1}$  for control P-replete cultures, whereas the required flow rate for P-limited cultures (30%  $\mu_{\max}$ ) was  $0.15 \text{ ml min}^{-1}$ .



**Figure 5-2: Mean maximum growth rate ( $\pm$ SEM,  $n=3$  except day 2:  $n=2$ ) of *Skeletonema marinoi* in the chemostat.**

### 5.2.2 Copepod Reproduction Experiments

Females of *Calanus helgolandicus* were collected weekly from the Northern Adriatic Sea and acclimated for several days on *Prorocentrum minimum*. Different populations of females were thus used in the different replicates, which were carried out over the course of several months. Individual females were incubated separately in 100-ml crystallizing dishes with an etched grid on the bottom, containing 0.22- $\mu$ m filtered seawater to which the necessary aliquot of *S. marinoi* collected from the outflow of the chemostat was added to reach 1 mg l<sup>-1</sup> food carbon concentrations ( $\sim 30,000$  cells ml<sup>-1</sup>). Females were transferred into fresh food medium daily with a wide-mouth glass pipette and the eggs and faecal pellets produced in 24 h counted under a dissecting microscope. Eggs were incubated for a further 48 h before fixation in 20% ethanol and counting of hatched nauplii, abnormal

nauplii, and nonviable eggs. Crystallizing dishes containing females and eggs were kept in a culture chamber on a 12h:12h light:dark cycle at 20°C. All in all, two replicates of two-week incubation experiments for P-limited cultures and three replicates of two-week incubation experiments for P-replete cultures were carried out in collaboration with Ylenia Carotenuto (SZN, Napoli).

### **5.2.3 Analysis of Lipoyxygenase Activity and Oxylin Production**

Samples for chemical analyses were taken from the chemostat and the outflow at regular intervals from both treatments during the two-week incubation experiments to analyze lipoyxygenase (LOX) metabolism in P-limited and P-replete cultures and to control whether the outflow was representative of the chemostat culture (Table 5-1). Phytoplankton samples were collected via the sterile syringe, concentrated by centrifugation (1000×g, 10 min, 4°C), frozen in liquid nitrogen, and stored at -80°C until analysis. Samples were analyzed for oxylin production through extraction with organic solvents and mass spectrometric analyses of extracts as described in Chapter 2 (2.2.4). Since collected sample amounts were small, only a few samples were analyzed for LOX activity by the colorimetric and the polarographic assays as described in Chapters 2 (2.2.3) and 3 (3.2.2), respectively. LOX activity measured in the colorimetric assay was defined as the difference in fatty acid hydroperoxide (FAH) concentrations between samples and blanks, without considering the addition of eicosapentaenoic acid (EPA).

**Table 5-1: Sampling of the chemostat and the outflow bottle of P-limited and P-replete *Skeletonema marinoi* culture during the course of the replicate experiments 1-3. Highlighted samples produced detectable amounts of aldehydes (see 5.3.2.1).**

P-status	Replicate	Day	Sample	P-status	replicate	Day	Sample
limited	1	0	Outflow	replete	1	7	Chemostat
		7	Chemostat			9	Outflow
		10	Outflow			14	Chemostat
		15	Outflow		2	0	Outflow
	2	0	Chemostat		3	1	Outflow
		1	Chemostat			1	Chemostat
		14	Chemostat			8	Outflow
						12	Chemostat
						15	Outflow
						15	Chemostat

**5.2.4 Analysis of Particulate Carbon, Nitrogen, and Phosphorus**

To control for P-limitation in chemostat cultures, culture samples for particulate carbon (POC), nitrogen (PON), and phosphorus (POP) were filtered onto acidified pre-combusted (450°C, 4 h) GF/F filters and stored at -80°C until analysis on a CHN elemental analyzer for POC and PON (FlashEA 1112 Series, ThermoQuest). Organic phosphorus (POP) was measured colorimetrically in an autoanalyzer (Flowsys, Systea) after decomposition in 1% potassium persulfate solution for 30 min at 120°C (Pujo-Pay and Raimbault 1994). Sample collection and preparation were carried out by Raffaella Casotti (SZN, Napoli) and measurements performed by Francesca Margiotta (SZN, Napoli). Two replicate samples

were analyzed for each experiment and the values pooled leading to four replicates for P-limited and six replicates for P-replete alga.

### 5.3 Results

#### 5.3.1 Nutrient Status

As expected, the content of particulate organic phosphorus was significantly lower in P-limited cells than P-replete cells of *Skeletonema marinoi* (t-test:  $t=4.19$ ,  $df=8$ ,  $p=0.0030$ ) leading to a high N/P-ratio of P-limited alga ( $33.9\pm10.5$ ) (Table 5-2). This N/P-ratio was significantly higher than the N/P-ratio of P-replete alga (t-test:  $t=3.64$ ,  $df=8$ ,  $p=0.0066$ ).

**Table 5-2: Mean values ( $\pm$ SEM; P-replete:  $n=6$ , P-limited:  $n=4$ ) for particulate organic carbon (POC), nitrogen (PON), and phosphorus (POP) content normalized for cells [ $\mu\text{g cell}^{-1}$ ] and C:N- and N:P-ratios in *Skeletonema marinoi* grown under P-replete and P-limited conditions.**

Parameter	P-replete	P-limited	t-test
POC [ $\mu\text{g cell}^{-1}$ ]	$19.3\pm2.2$	$42.0\pm6.0$	$p=0.0034$
PON [ $\mu\text{g cell}^{-1}$ ]	$2.0\pm0.2$	$2.4\pm0.2$	$p>0.05$
POP [ $\mu\text{g cell}^{-1}$ ]	$0.37\pm0.05$	$0.13\pm0.02$	$p=0.0030$
C:N	$8.4\pm0.6$	$14.8\pm1.1$	$p=0.0005$
N:P	$15.4\pm1.2$	$33.5\pm6.0$	$p=0.0066$

The N/P-ratio in P-limited alga was higher than the Redfield ratio of N/P=16, indicating that cells of *S. marinoi* grown at low dilution rates (30%  $\mu_{\max}$ ) were indeed limited in phosphorus. The N/P-ratio of *S. marinoi* grown at high dilution rates (90%  $\mu_{\max}$ ) was also close to the Redfield ratio at  $15.1 \pm 1.7$ .

### 5.3.2 Oxylipin Production

#### 5.3.2.1 Aldehyde Production

Aldehydes were detectable in only three of the 17 samples analyzed (highlighted in Table 5-1). In all three samples, the only aldehyde detected was octadialenal, which derives from C<sub>16</sub>-fatty acids.

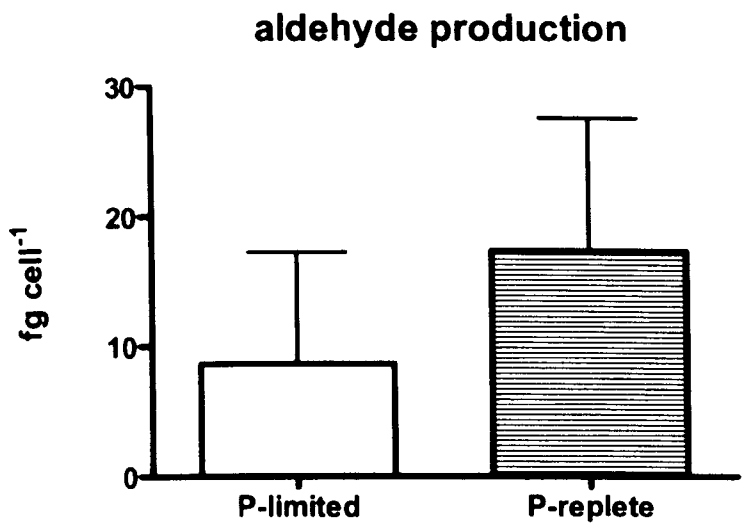


Figure 5-3: Mean aldehyde production (+SEM; P-limited: n=5, P-replete: n=7) normalized for cells [fg cell<sup>-1</sup>] in P-limited and P-replete cultures of *Skeletonema marinoi*. Data for samples of chemostat and outflow bottles were pooled.

Phytoplankton samples showed no significant difference in aldehyde production due to P-limitation (t-test:  $p>0.05$ ) (Figure 5-3). However, considering that only two samples of the P-replete culture and only one of the P-limited culture produced any detectable amount of aldehydes, these data should be regarded as only indicative.

5.3.2.2 *Production of Oxylipins Other than Aldehydes*

Also the production of oxylipins other than aldehydes was low and variability was high among replicate treatments. No derivatives of C<sub>20</sub>-fatty acids were detected in the samples. Only low amounts of the C<sub>16:4</sub>-hydroxy-acid were present and in some cases low amounts of C<sub>16:3</sub>-hydroxy-acid and C<sub>16:3</sub>-epoxyalcohol were also detectable. Neither aldehyde production nor the production of other oxylipins demonstrated metabolism of C<sub>20</sub>-fatty acids under the conditions of this experiment.

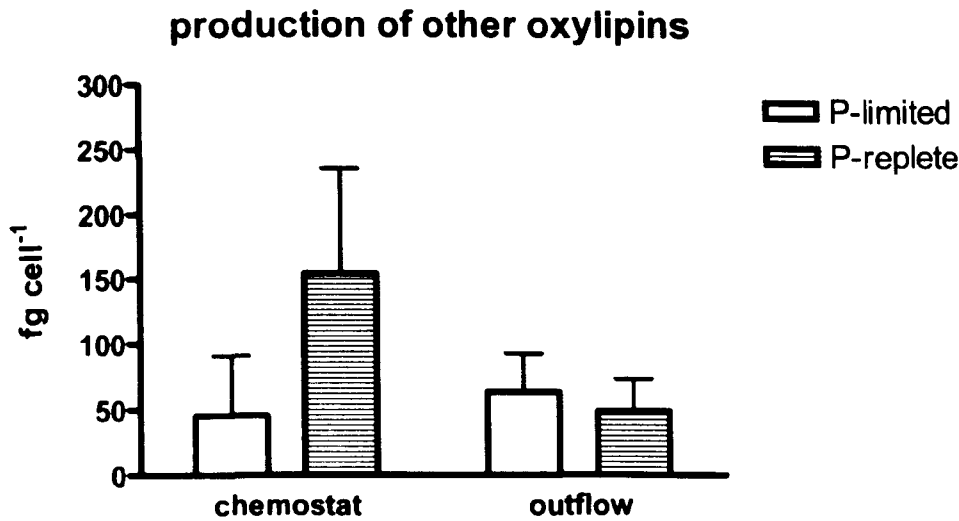


Figure 5-4: Mean production of oxylipins other than aldehydes (+SEM; P-limited chemostat: n=3, P-limited outflow: n=2, P-replete chemostat: n=3, P-replete outflow: n=4) normalized for cells [fg cell<sup>-1</sup>] in chemostat and outflow bottles of P-limited and P-replete cultures of *Skeletonema marinoi*.

When the chemostat and outflow samples were considered together for each treatment, the production of oxylipins other than aldehydes did not differ significantly between P-replete and P-limited cultures (t-test:  $p>0.05$ ) (Figure 5-4).

5.3.2.3 Oxylipin Production

Since values for aldehyde production were low, looking at the production of aldehydes and oxylipins other than aldehydes together gave the same picture as Figure 5-4 (Figure 5-5). There were no significant differences in oxylipin production due to P-limitation in *Skeletonema marinoi* in the present experiment (t-test:  $p>0.05$ ).

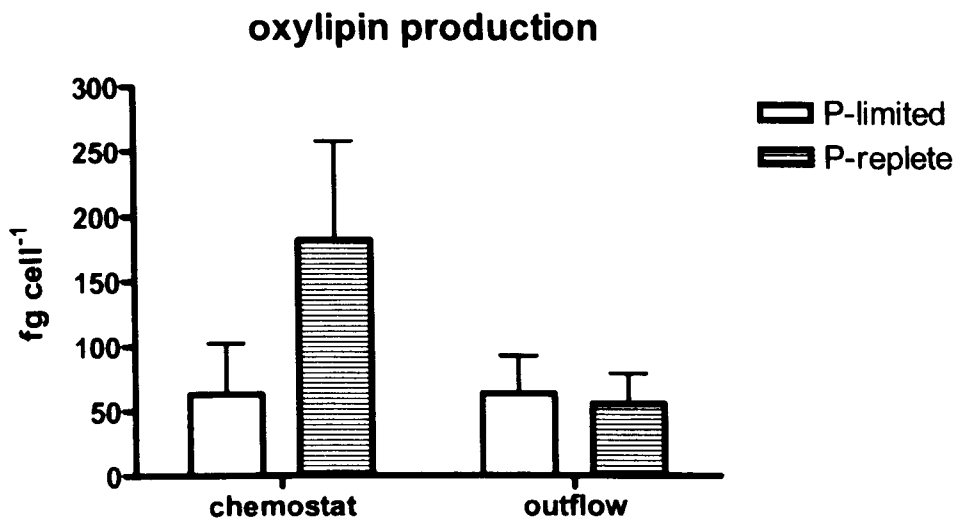
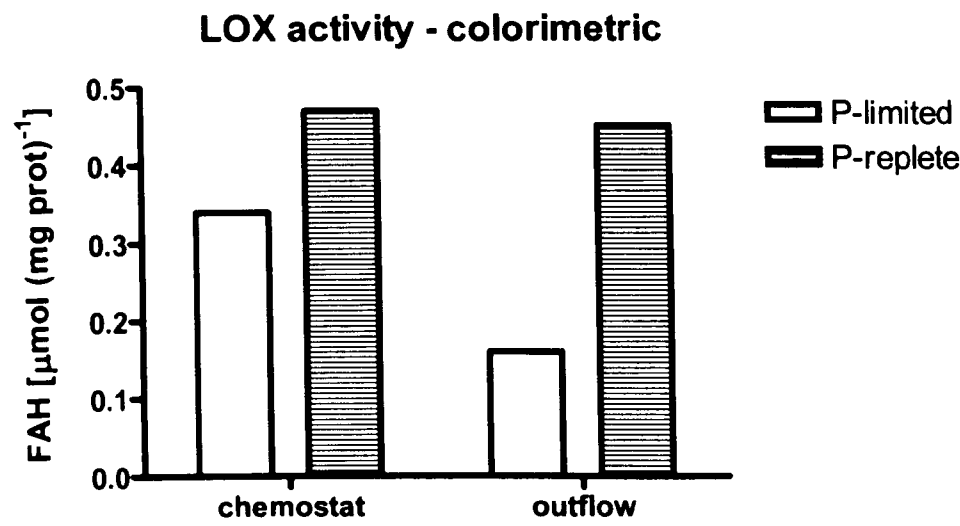


Figure 5-5: Mean oxylipin production (+SEM, P-limited chemostat: n=3, P-limited outflow: n=2, P-replete chemostat: n=3, P-replete outflow: n=4) normalized for cells [fg cell<sup>-1</sup>] in chemostat and outflow bottles of P-limited and P-replete cultures of *Skeletonema marinoi*.



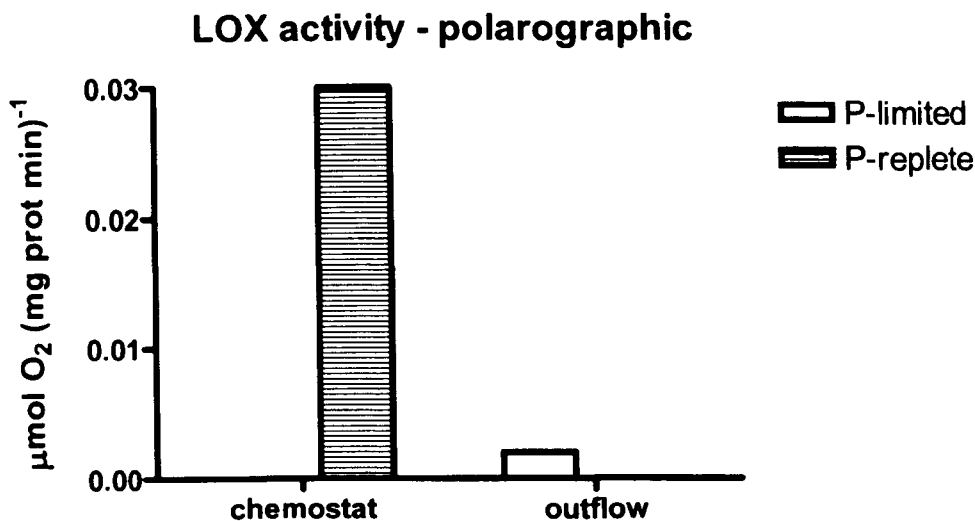
### 5.3.3 Lipoxygenase Activity

Lipoxygenase (LOX) activity seemed weaker in the P-limited than in the P-replete cultures in the chemostat as well as the outflow bottle (Figure 5-6). However, since only single measurements were carried out, statistical analyses were not possible.



**Figure 5-6: Fatty acid hydroperoxide (FAH) production  $[\mu\text{mol (mg prot)}^{-1}]$  (n=1) determined with the colorimetric assay in chemostat and outflow bottles of P-limited and P-replete cultures of *Skeletonema marinoi*. FAH production was defined as the difference in FAH concentrations between cell lysates after 20 min and blanks.**

Data were scarce for polarographic measurements with only one sample analyzed for P-limited cultures and only one for P-replete cultures. However, also in this assay, the results indicated that P-replete cultures had stronger LOX activity (Figure 5-7).



**Figure 5-7:** Oxygen consumption rate [ $\mu\text{mol O}_2 \text{ (mg prot min)}^{-1}$ ] upon EPA-addition (0.4 mM) to cell lysate ( $n=1$ ) determined with the polarographic assay at pH=8.15 in the chemostat bottle of P-replete culture and the outflow bottle of P-limited culture of *Skeletonema marinoi*.

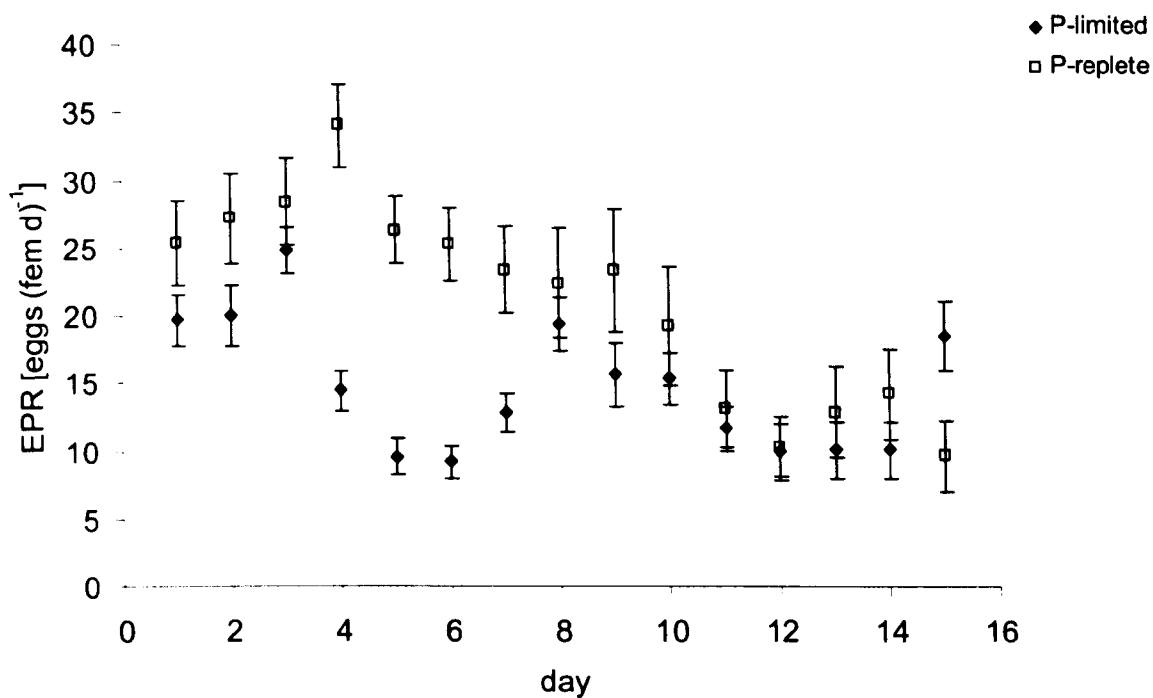
### 5.3.4 Copepod Reproduction

Two replicates of copepod incubation experiments for P-limited culture were carried out for 15 days with 14 and 15 *Calanus helgolandicus* females, respectively. Only 64% were still alive after day 8 in the first replicate, whereas survival was improved in the second replicate with 80% of females still alive from day 11 onwards (Table 5-3). The first P-replete replicate was carried out with 13 females for 15 days with survival high at 85% as of day 11. The second replicate, however, was stopped after 12 days because only one of the initial 10 females had survived up to day 12 and was no longer producing eggs. Therefore a third replicate was carried out for 15 days with only four females, of which all survived until the end of the experiment. Data of all replicates for females incubated with P-replete cultures and for females incubated with P-limited cultures, respectively, were pooled.

**Table 5-3: Parameters (Number of days, initial number of females and survival rate of females [%]) of the incubation experiments carried out with females of *Calanus helgolandicus* feeding on P-limited and P-replete cultures of *Skeletonema marinoi*.**

Treatment	Replicate	No. days	Initial no. females	Survival [%]
P-limited	1	15	14	64 (day8)
	2	15	15	80 (day11)
P-replete	1	15	13	85 (day11)
	2	12	10	10 (day12)
	3	15	4	100

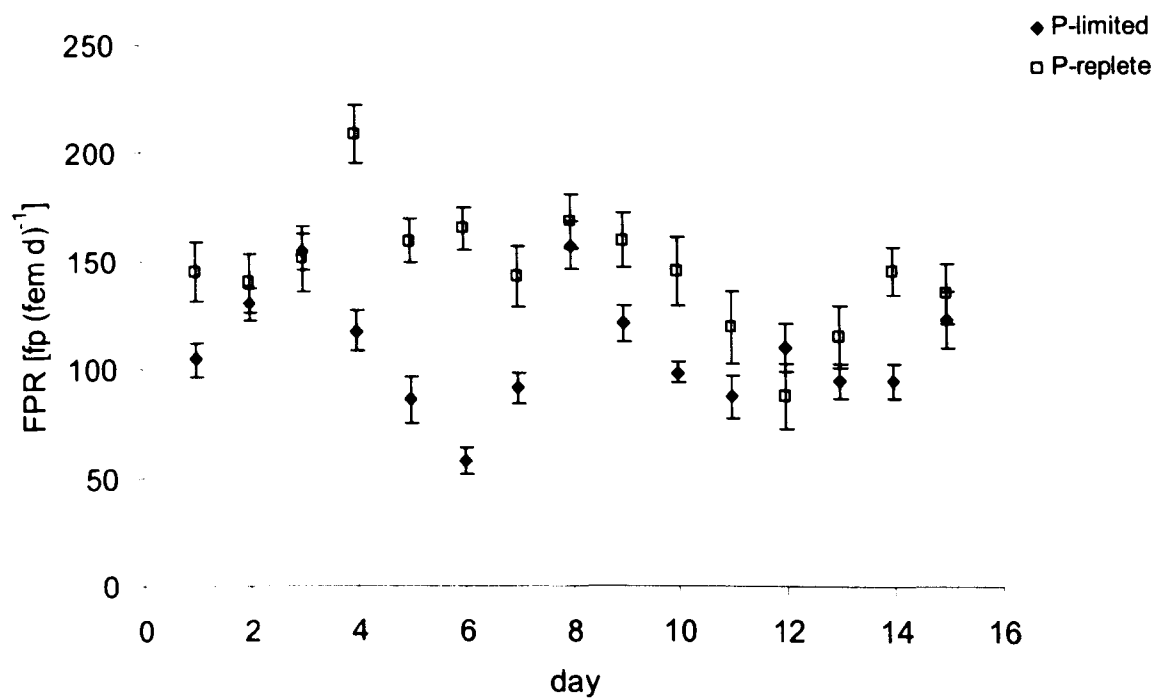
Egg production rates (EPR) were more or less constant when *C. helgolandicus* fed on P-limited cultures with an average ( $\pm$ SEM) of  $15\pm1$  eggs (female day)<sup>-1</sup>, whereas feeding on P-replete cultures resulted in a higher overall average of  $21\pm2$  eggs (female day)<sup>-1</sup> (Figure 5-8).



**Figure 5-8: Mean egg production rates (EPR) ( $\pm$ SEM,  $n=15-29$ , depending on day and treatment) [eggs (fem d)<sup>-1</sup>] of *Calanus helgolandicus* females feeding on P-limited and P-replete cultures of *Skeletonema marinoi* from outflow bottles of the chemostat set-up.**

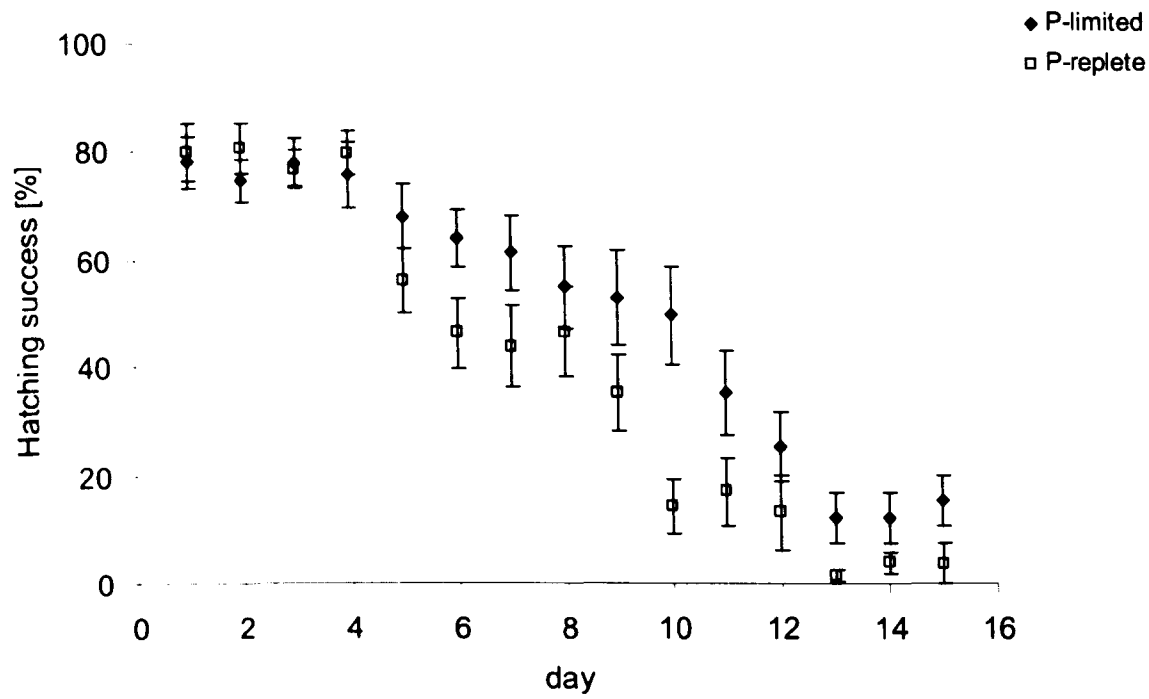
EPR of females feeding on P-replete cultures was modulated, being higher at the beginning of the experiment with a steady decrease over time, whereas EPR of females feeding on P-limited cultures was more or less constant over the entire experiment. Mean overall EPR was significantly higher in females feeding on P-replete compared to P-limited *S. marinoi* (paired t-test:  $t=3.35$ ,  $df=14$ ,  $p=0.0048$ ).

Also faecal pellet production rate (FPR) was significantly higher in females feeding on P-replete compared to P-limited cultures (paired t-test:  $t=4.13$ ,  $df=14$ ,  $p=0.0010$ ) (Figure 5-9).



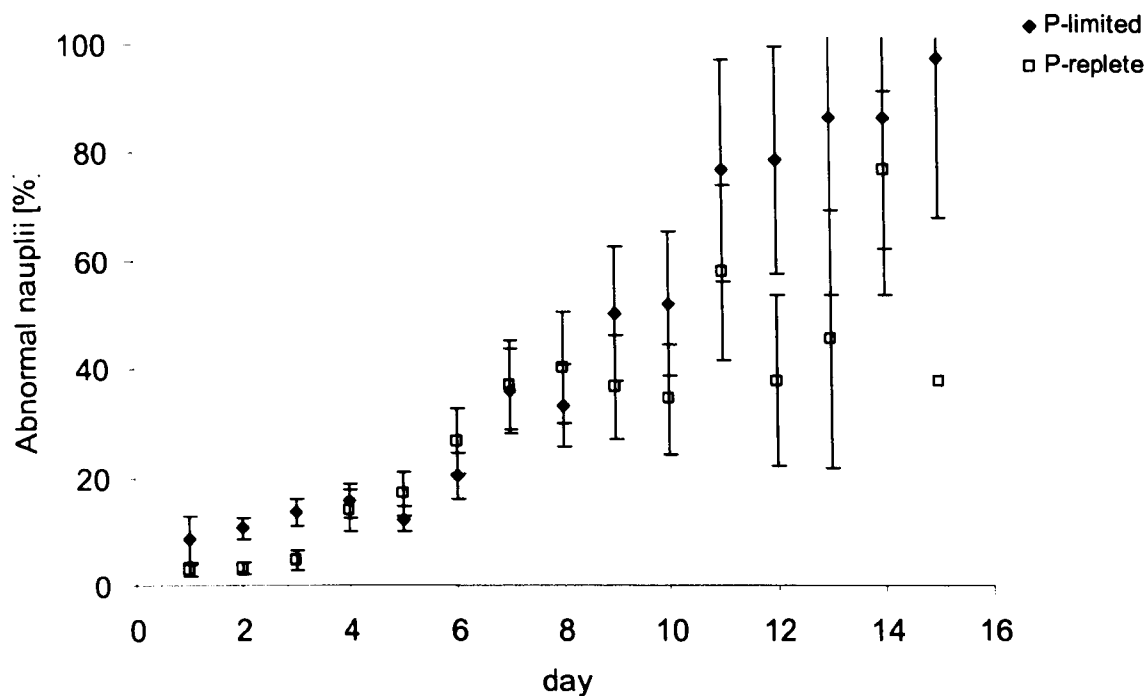
**Figure 5-9:** Mean faecal pellet production rates (FPR) ( $\pm$ SEM,  $n=15-29$  depending on day and treatment) [fp (fem d)<sup>-1</sup>] of *Calanus helgolandicus* females feeding on P-limited and P-replete cultures of *Skeletonema marinoi* from outflow bottles of the chemostat set-up.

FPR did not vary greatly with time over the course of the experiment with an average ( $\pm$ SEM) of  $146\pm7$  faecal pellets (female day)<sup>-1</sup> for females feeding on P-replete cultures compared to  $109\pm7$  faecal pellets (female day)<sup>-1</sup> for females feeding on P-limited cultures (Figure 5-9).



**Figure 5-10: Mean hatching success ( $\pm$ SEM, n=13-29 depending on day and treatment) [%] of eggs produced by *Calanus helgolandicus* females feeding on P-limited and P-replete cultures of *Skeletonema marinoi* from outflow bottles of the chemostat set-up.**

Hatching success of eggs produced by females feeding on P-limited cultures was significantly higher than that of females feeding on P-replete cultures (paired t-test:  $t=3.85$ ,  $df=14$ ,  $p=0.0018$ ) (Figure 5-10). However, hatching success in general was impacted strongly in both treatments with hatching success below 50% after 9 days of feeding on P-replete cultures and after 10 days of feeding on P-limited cultures.



**Figure 5-11: Mean percentage of abnormal nauplii ( $\pm$ SEM,  $n=3-29$  depending on day and treatment) [%] hatched from eggs produced by *Calanus helgolandicus* females feeding on P-limited and P-replete cultures of *Skeletonema marinoi* from outflow bottles of the chemostat set-up.**

The percentage of abnormal nauplii increased strongly in both treatments with time (Figure 5-11). The percentage of abnormal nauplii was significantly higher in nauplii hatched from copepod females feeding on P-limited culture than in nauplii hatched from copepod females feeding on P-replete culture (paired t-test:  $t=2.73$ ,  $df=14$ ,  $p=0.016$ ). Apparently, P-limited cultures induced stronger abnormality in copepod nauplii towards the end of the experiment compared to P-replete cultures.

## 5.4 Discussion

Neither oxylipin production, nor lipoxygenase (LOX) activity measurements such as fatty acid hydroperoxide (FAH) production determined with the colorimetric assay or oxygen consumption rate measured polarographically, demonstrated increased oxylipin metabolism in P-limited cultures of *Skeletonema marinoi* grown in the chemostat. This contradicted the hypothesis, that P-limitation should act as a stress factor, increasing LOX activity and therefore production of fatty acid derived oxygenated metabolites, as was demonstrated by Ribalet *et al.* (2007b). An increase in oxylipin production due to phosphorus stress could not be confirmed in the present experiment. However, it is interesting to note that this strain of *S. marinoi* was apparently active at pH=6.0 under the present experimental conditions because an increase in FAH concentrations from the blanks to the samples could be measured in the colorimetric assay.

Analysis of phosphorus and nitrogen content in the cells showed that although the N/P-ratio in P-limited alga was significantly higher than that of P-replete alga, *S. marinoi* grown at high dilution rates was also close to P-limitation. This may explain the lack of difference in oxylipin production between the two treatments, especially if the influence of P-limitation on LOX metabolism is not continuous, but due to a threshold value. Alternatively, it is possible that P-limitation did not have a strong effect on LOX metabolism and other stress factors may be more effective at inducing an increase in fatty acid oxidation. Indeed, in the work of Ribalet *et al.* (2009), silica-limitation was identified as a stronger stress factor and induced a much stronger increase in aldehyde production than phosphorus-limitation. It may therefore be useful to repeat these experiments with silica-limited culture medium to observe a more significant effect on LOX metabolism.



Although there were no significant differences in oxylin production between the chemostat bottle and the outflow culture, LOX activity assays indicated increased activity in the chemostat bottle. The lack of agreement between chemostat and outflow culture may be due to changing nutrient regimes from the chemostat to the outflow, because cells washed into the outflow bottle are no longer under a constant dilution rate. One may suppose that in the outflow, cells should be even more nutrient-stressed because of the lack of fresh culture medium. However, in the outflow some recycling of nutrients may be taking place, for example by bacteria, although care was taken to keep chemostats axenic and the outflow culture was left in the outflow bottle at maximum for three days. Axenity may be improved upon by a different method of sampling. Instead of removing samples by aspiration through a syringe and thereby having a direct contact with the chemostat, in the work of Caperon the air supply tube was used for sampling (Caperon 1968). This was a two-way tube in which the culture was made to rise and flow out by previously clamping off the outflow and increasing the pressure inside the chemostat through increased air-flow. After sampling and opening of the outflow, the tube was once again used to aerate the culture avoiding culture growth in the tube. This method provided a very sterile way to sample chemostats (Caperon 1968). However, in our case the culture chamber was fitted with a stirring device that had to move freely, thereby not allowing an air-tight closure of the chemostat, making this sampling option not feasible. In the present experiments continuous sampling for chemical analyses were required to control for a continuous metabolism of the alga during the entire two-week incubation experiment. Therefore a different chemostat set-up may be advisable for the present experiments to decrease contamination risk by allowing a different sampling method. It would be preferable to incubate copepods directly with algal culture from the chemostat, because culture conditions are more closely controlled in the chemostat bottle and cells are

probably impacted more strongly by nutrient limitation. However, since incubation experiments needed to be carried out over several weeks to induce an observable effect on copepod reproduction, using the actual chemostat to feed females was impractical because it would have required large volumes and continuous adjusting of the flow rate upon volume changes.

Egg production rates (EPR) and faecal pellet production rates (FPR) were significantly higher in P-replete treatments. This may have been due to a higher nutritional value of P-replete cultures. However, when replicates were considered individually, differences for EPR and FPR overlapped. EPR and FPR were highest in the third replicate carried out with P-replete culture and lowest in the first replicate carried out with P-replete culture. Therefore these two parameters were probably not dependent on the nutritional status of food alga, but rather due to internal factors. Both parameters were probably more indicative of general female health and probably not related to incubation conditions. Since each replicate was carried out with a fresh batch of females most likely with different histories, intrinsic factors may have been different even though females were acclimated for several days before starting incubation experiments. EPR seemed to correlate positively to FPR, hence to feeding. Females consuming more microalgae were producing more eggs in consequence.

The two parameters for determining the effect of diatoms on copepod reproduction, hatching success and the percentage of abnormal nauplii did not correspond. Whereas hatching success was significantly higher in P-limited culture, which was contrary to expectations, the percentage of abnormal nauplii was significantly lower in P-replete cultures, as expected. Apparently, both treatments had a similar deleterious effect on copepod reproduction. This corresponded to the fact that P-limited and non-limited algae

were producing more or less the same amount of oxylipins, having therefore similar “toxicity” and a similar negative effect. In fact, both treatments strongly reduced copepod hatching success to 50% after 9 and 10 days of feeding on P-replete and P-limited cultures, respectively.

## 5.5 Conclusions

In the present chemostat experiment, phosphorus-limitation did not induce an increased production of oxylipins in *Skeletonema marinoi*, even though N/P-ratios were significantly higher in algae grown under low compared to high dilution rates. When *Calanus helgolandicus* females fed on the different treatments, hatching success of nauplii resulted higher for females feeding on P-limited compared to P-replete alga, which was contrary to expectations. However, the percentage of abnormal nauplii produced by females was higher with females feeding on P-limited alga. In general, both treatments had a strong effect on copepod reproductive success probably due to the similar production of oxylipins.

## 6 Growth-phase Modulated Lipxygenase Activity in *Pseudo-nitzschia delicatissima*

(Results on the presence of lipxygenase activity in *Pseudo-nitzschia delicatissima*, its modulation during different growth phases, and the characterization of oxylipin compounds produced, are published in d'Ippolito *et al.* 2009)

Blooms of *Pseudo-nitzschia delicatissima* have been found to negatively impact copepod reproductive success at sea. Therefore, a thorough study of the lipxygenase (LOX) metabolism of this diatom species was carried out with a special emphasis on the variations during various growth phases. Oxylipins were characterized and quantified during five distinct growth phases, early exponential, mid exponential, late exponential, stationary, and declining phase. LOX metabolism was also analyzed by the polarographic and colorimetric LOX activity assays in each of the growth phases. *P. delicatissima* produced three major oxylipins, 15(*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*Z*,17*Z*)-eicosapentaenoic acid (15*S*-HEPE), 13,14-*threo*-13*S*-hydroxy-14*S*,15*S*-*trans*-epoxyeicosa-5*Z*,8*Z*,11*Z*,17*Z*-tetraenoic acid (13,14-HEpETE) and 15-oxo-5*Z*,9*E*,11*E*,13*E*-pentadecatetraenoic acid. Whereas the novel oxo-acid was produced only upon entry into the stationary phase, 15*S*-HEPE and 13,14-HEpETE were produced continuously with an increase in the latter phases of culture growth. The intensification of oxylipin metabolism at the end of culture growth was corroborated by an increase in LOX activity identifiable polarographically through an increase in oxygen consumption rate, as well as colorimetrically by an increased production of fatty acid hydroperoxides (FAHs). These results may suggest the involvement of oxylipins in regulating culture growth.

## 6.1 Introduction

In the present study, the pennate diatom *Pseudo-nitzschia delicatissima* was examined for oxylipin metabolism. *P. delicatissima* is a cosmopolitan species (Hasle 2002), forming blooms in coastal waters (Lundholm *et al.* 2004; Quijano-Scheggia *et al.* 2008) and upwelling regions (Iriarte and Fryxell 1995). Low hatching success of copepod eggs in the field has been reported during blooms of this diatom (Miralto *et al.* 1999; Miralto *et al.* 2003) and ascribed to the production of polyunsaturated aldehydes (PUAs) (Miralto *et al.* 1999). Therefore this species was judged an interesting candidate for a closer examination of oxylipin metabolism. Furthermore, possible variations in oxylipin metabolism during the various growth phases were of interest.

In culture, growth of planktonic diatoms is generally characterized by a short lag-phase after initial inoculation, followed by an exponential growth phase with rapid growth until rates level off towards a stationary phase with more or less constant cell concentrations. The length of this stationary phase is apparently species-specific. In *Skeletonema marinoi*, stationary phase could last for one (personal observations) to two weeks (Vidoudez and Pohnert 2008), whereas *P. delicatissima* had an extremely short stationary phase lasting a maximum of two days (see data below). The reason for rapid decline of cell concentrations, the so-called “crashing” of a diatom culture, is still poorly understood (Franklin *et al.* 2006). Although in natural systems primary production by diatoms is often balanced by losses due to grazing and sinking (Horn and Horn 2000), carbon budgets are not always concordant and “enigmatic losses” have been described (Walsh 1983). Viruses (Suttle and Chan 1990; Brussaard 2004; Suttle 2005) and bacteria have been suggested as important factors in phytoplankton cell death. Bacteria can impact algal growth either by cell lysis or through competition for nutrients (Cole 1982). Another possible reason for cell

lysis is attack by parasites e. g. flagellates (Kuehn 1998) or fungi (Holfeld 1998). Whereas all of these mechanisms may be active in nature, they are probably not relevant in a small-volume axenic culture, therefore the real reason for rapid cell lysis in culture remains unclear.

Recently, it has been found that diatoms release metabolites into the surrounding culture medium depending on the growth phase in culture (Vidoudez and Pohnert 2008; Barofsky *et al.* 2009). The former authors demonstrated the specific release of two aldehydes from *S. marinoi* at the end of the stationary growth phase shortly before the decline of the culture, whereas the latter authors determined an entire metabolomic pattern that showed phase-dependent quantitative and qualitative changes. This modulated production suggests a signalling function for oxylipins akin to quorum sensing in bacteria (Falciatore and Bowler 2002; Barofsky *et al.* 2009), possibly leading to a co-ordinated “crashing” of the culture. In bacteria, quorum sensing has become an established concept during the last decade (Fuqua *et al.* 1994; Williams *et al.* 2007). Quorum sensing is a density-dependent signalling process resulting from the production and diffusion of small molecules by bacteria, whose concentrations increase proportionally to population growth until reaching a threshold level at which the population responds (Williams *et al.* 2007). Therefore it is tempting to suggest a similar mechanism in phytoplankton, which would allow these unicellular organisms to respond in a coordinated “multicellular-like” way.

The concept of oxylipins as inducers of autocatalytic cell death has been suggested by Vardi *et al.* (2006) who proposed aldehydes as a possible signal for bloom-termination due to increased stress. However, programmed cell death is a poorly understood and sparsely researched phenomenon in phytoplankton (Franklin *et al.* 2006). Oxylipin production may be an example of cell to cell signalling leading to rapid decline in cell numbers (Vardi *et*

*al.* 2006; Vidoudez and Pohnert 2008), a process possibly induced by nutrient stress (Ribalet *et al.* 2007b). To try and validate this hypothesis, *P. delicatissima* was analyzed for lipoxygenase (LOX) activity and oxylipin production during the development and decline of a culture. Changes in these two parameters were followed to determine variations in fatty acid oxidative metabolism depending on the phase of culture growth.

## 6.2 Materials and Methods

### 6.2.1 Sample Collection

*Pseudo-nitzschia delicatissima* (Strain SZN B321; IST sequence identical to AL-24 GenBank DQ813830 (Amato *et al.* 2007)) was cultured in 10-litre polycarbonate bottles on autoclaved f/2 medium prepared from 0.22- $\mu\text{m}$  filtered oligotrophic seawater (Guillard 1975). Cultures were bubbled gently with sterile (0.22- $\mu\text{m}$  filtered) ambient air and grown at 20°C under a 12h:12h light:dark cycle (100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) in a climate chamber. Initial cell concentrations were 300-500 cells  $\text{ml}^{-1}$  upon inoculation and culture growth was followed by daily enumeration in a Sedgewick counting chamber. Samples were collected by centrifugation (1500 $\times$ g, 10 min, 4°C) in five distinct phases along the growth curve: early exponential (day 3 after inoculation), mid exponential (day 4 to 5), late exponential (day 6), stationary (day 7 to 8), and declining phase (day 9 onward). The obtained pellets were frozen in liquid nitrogen and kept at -80°C until analysis. Phytoplankton culturing and sample collecting were carried out by Marina Montresor and Carmen Minucci (SZN, Napoli).

Four complete replicate growth curves were run and the values integrated. Additionally, four samples for the initial phase (early exponential) and one for the declining phase were

collected and these samples considered in the mean value for each growth phase, eliminating outlier samples.

### 6.2.2 Sample Analysis

Modulation of oxylipin metabolism was determined by three parameters: production of oxylipins, oxygen consumption rate of cell lysates upon addition of exogenous fatty acids, and production of intermediate fatty acid hydroperoxides (FAHs) by cell lysates.

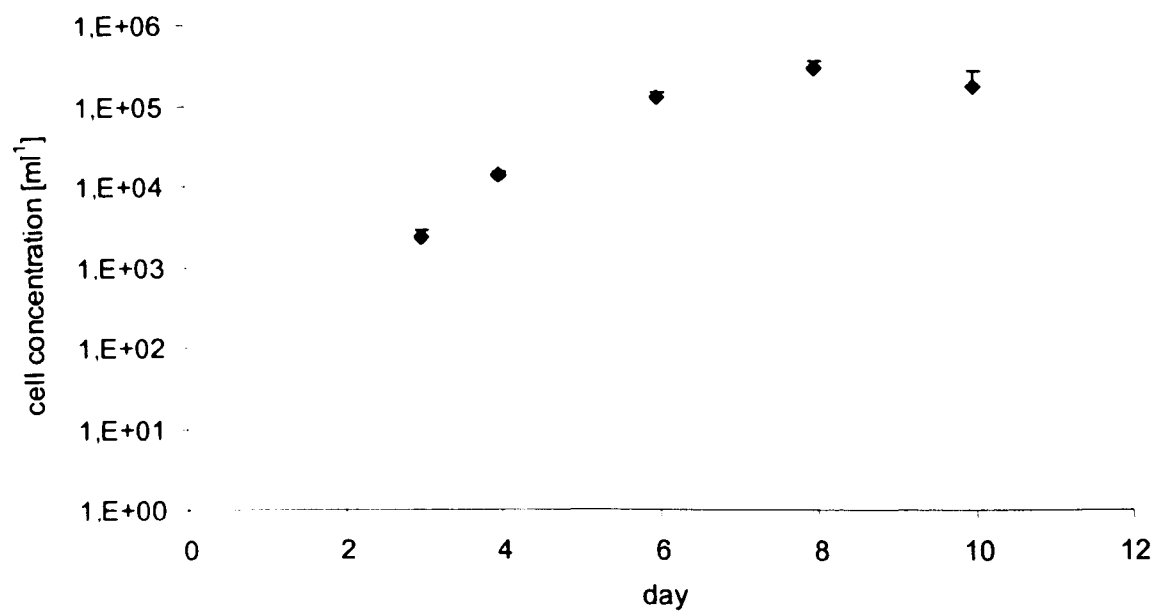
Cell lysates were prepared as described in Chapter 2 (2.2.2.2), except that pellets were suspended in 50 mM phosphate buffer (pH=6.5) instead of H<sub>2</sub>O<sub>deionised</sub>. Subsamples were removed from the cell lysates for protein determination and LOX activity assays as described in Chapters 2 (2.2.3, 2.2.6) and 3 (3.2.2). The colorimetric assay for these experiments was carried out by incubating cell lysate with the colorimetric agents without the addition of external fatty acids (EPA), because the endogenous pool of fatty acids should suffice for supporting LOX activity. Activity was therefore calculated as in Chapter 3, as the difference in FAH concentrations between the sample read after 20 min and the blank read immediately. The cell lysate was extracted and oxylipin production quantified on LC-MS/MS and GC-MS as described in Chapter 2 (2.2.4). Characterization of oxylipins was carried out by Giuliana d'Ippolito and Adele Cutignano (ICB-CNR, Pozzuoli). Stereochemistry of 15-HEPE was analyzed on chiral phase HPLC using a Chiralcel OD-H (4.6×250 mm, flow rate 1.5 ml min<sup>-1</sup>, UV detection at 236 nm) column eluted isocratically with hexane-isopropanol (98:2 v:v) (Fontana *et al.* 2007b).



## 6.3 Results

### 6.3.1 Growth Curve

The average growth curve was characterized by an exponential phase lasting 6 days at a rapid growth rate of  $1.7\text{ d}^{-1}$ . This was followed by a short stationary phase lasting a maximum of two days with peak cell concentrations of  $2.9\pm0.6\cdot10^5\text{ cells ml}^{-1}$  (Figure 6-1). After this brief stationary phase, cultures went into senescence with cell numbers declining sharply from day 8 onwards.



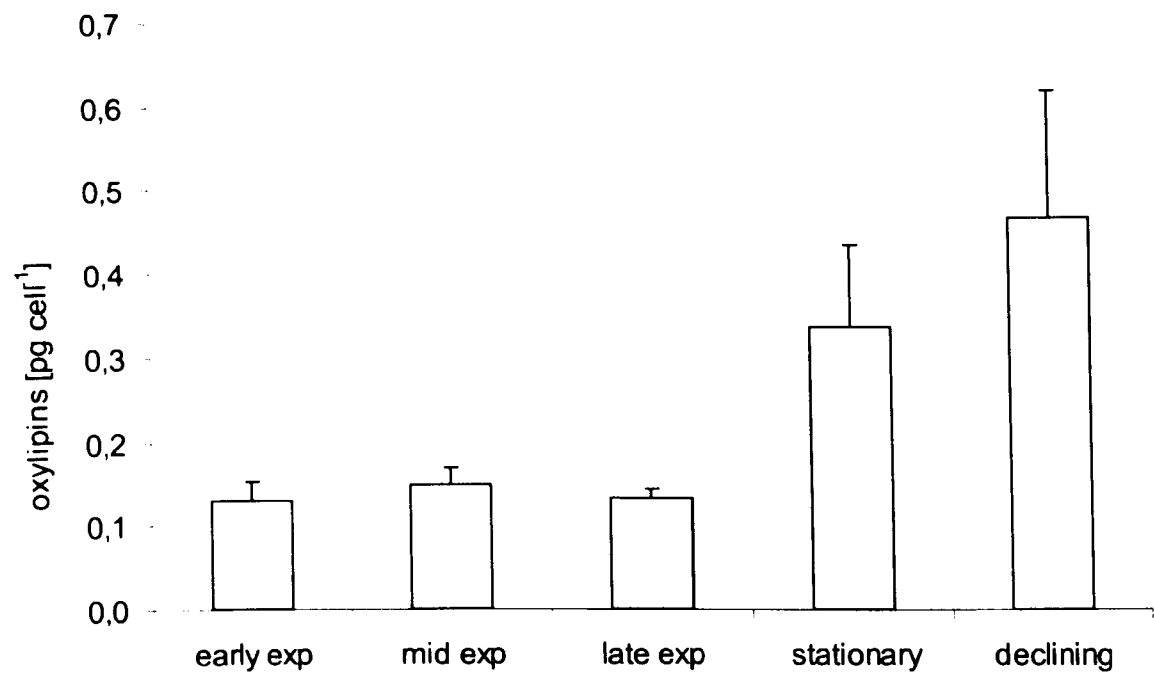
**Figure 6-1:** Mean cell concentrations ( $\pm$ SEM;  $n=3$  except day 3:  $n=5$ , day 10:  $n=4$ ) [ $\text{ml}^{-1}$ ] of *Pseudo-nitzschia delicatissima* during culture growth.

### 6.3.2 Oxylin Production

GC-MS analysis of cell extracts showed an absence of volatile aldehydes in this diatom species (see Appendix 4). LC-MS/MS analysis after methylation of the extract,

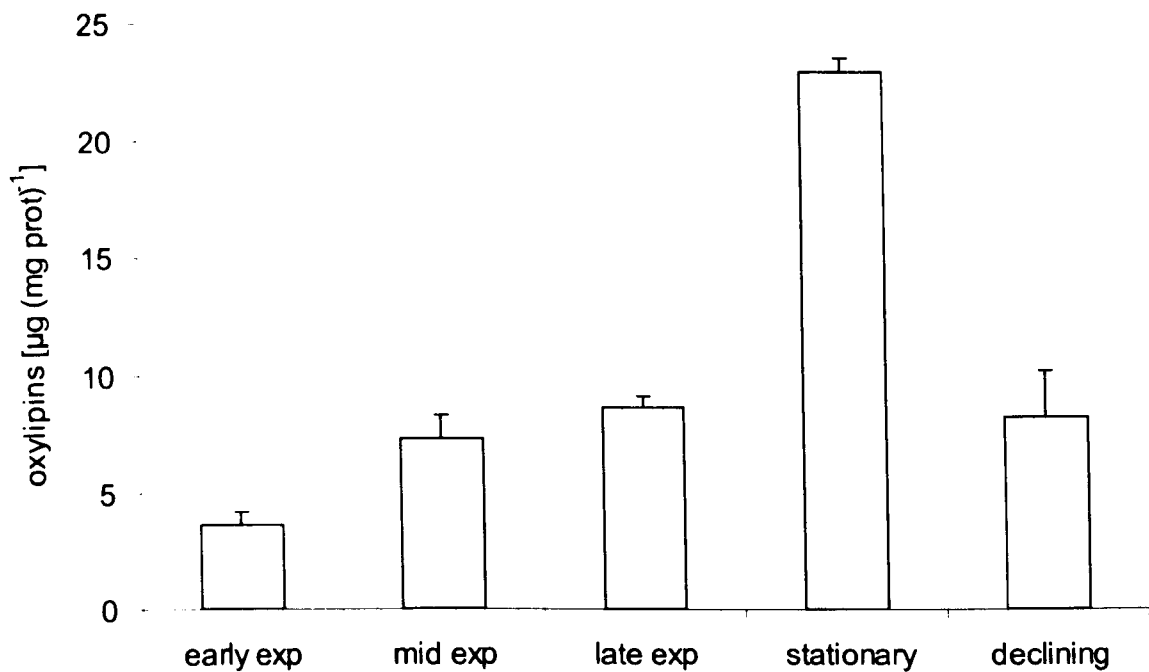
demonstrated the production of three major oxylipins by *Pseudo-nitzschia delicatissima*: 15(*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*Z*,17*Z*)-eicosapentaenoic acid (15*S*-HEPE), 13,14-*threo*-13*S*-hydroxy-14*S*,15*S*-*trans*-epoxyeicosa-5*Z*,8*Z*,11*Z*,17*Z*-tetraenoic acid (13,14-HEpETE) and 15-oxo-5*Z*,9*E*,11*E*,13*E*-pentadecatetraenoic acid. Absolute stereochemistry of 15*S*-HEPE was established as 99% by co-elution with authentic standards on chiral HPLC, whereas the *trans* configuration of the 14,15-epoxide ring of 13,14-HEpETE was assigned on the basis of the coupling constant between the H14 and H15 protons in NMR analysis.

Oxylipin production varied at different growth phases (Figures 6-2 and 6-3).



**Figure 6-2: Mean oxylipin production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for cells [pg cell<sup>-1</sup>] in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining).**

Oxylipin production per cell was constantly low during the entire exponential phase at  $0.14\pm0.01$  pg cell<sup>-1</sup>. Production increased significantly in the stationary phase to  $0.34\pm0.10$  pg cell<sup>-1</sup> (t-test between combined exponential phase (n=11) and stationary phase (n=3):  $t=3.94$ ,  $df=12$ ,  $p=0.002$ ) with a minor increase in the declining phase to  $0.47\pm0.15$  pg cell<sup>-1</sup> (t-test between stationary phase (n=3) and declining phase (n=4):  $p>0.05$ ) (Figure 6-2).



**Figure 6-3: Mean oxylipin production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for protein [µg (mg prot)<sup>-1</sup>] in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining).**

Also when oxylipin production was normalized for protein, the highest value could be found in the stationary phase with cells producing  $22.9\pm0.6$  µg oxylipins (mg prot)<sup>-1</sup> (Figure 6-3). This was a significantly higher oxylipin production compared to all other phases (one-way ANOVA:  $F=19.65$ ,  $r^2=0.91$ ,  $p=0.0003$ ).

6.3.3 Lipoxygenase Activity

6.3.3.1 Polarographic Assay

Correspondingly, lipoxygenase (LOX) activity as determined polarographically also seemed highest in the stationary phase with an increase in oxygen consumption rate of 0.013  $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$  upon the addition of external eicosapentaenoic acid (EPA) to cell lysate (Figure 6-4). Differences between phases, however, were not statistically significant (one-way ANOVA:  $p>0.05$ ).

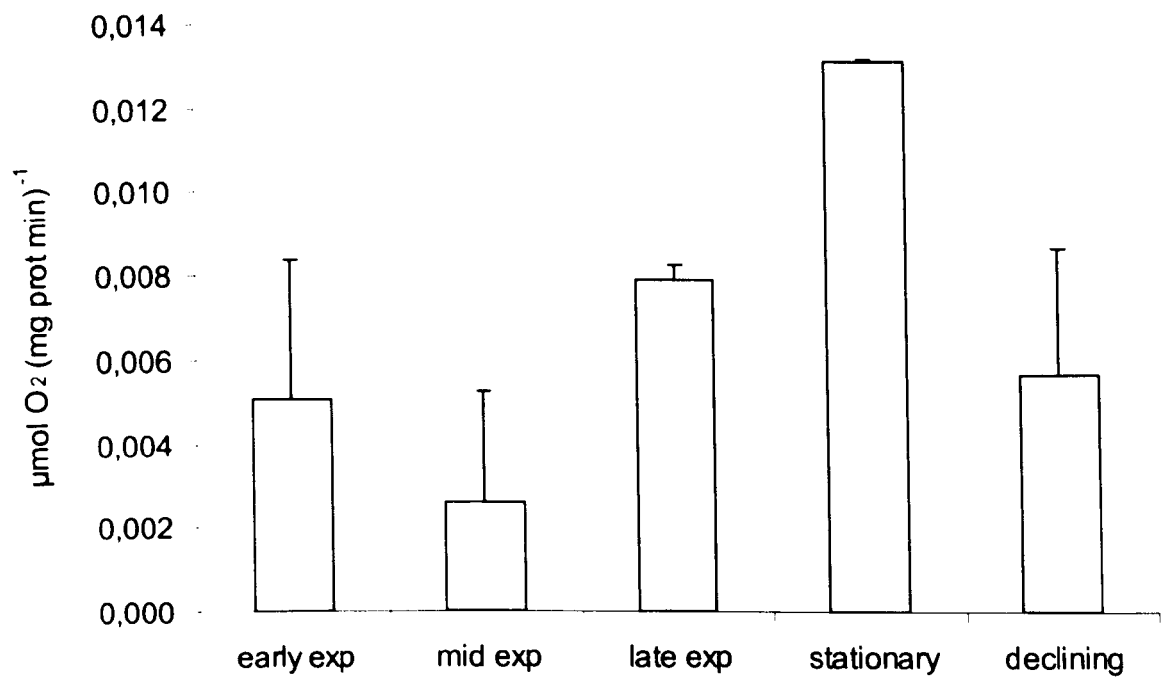
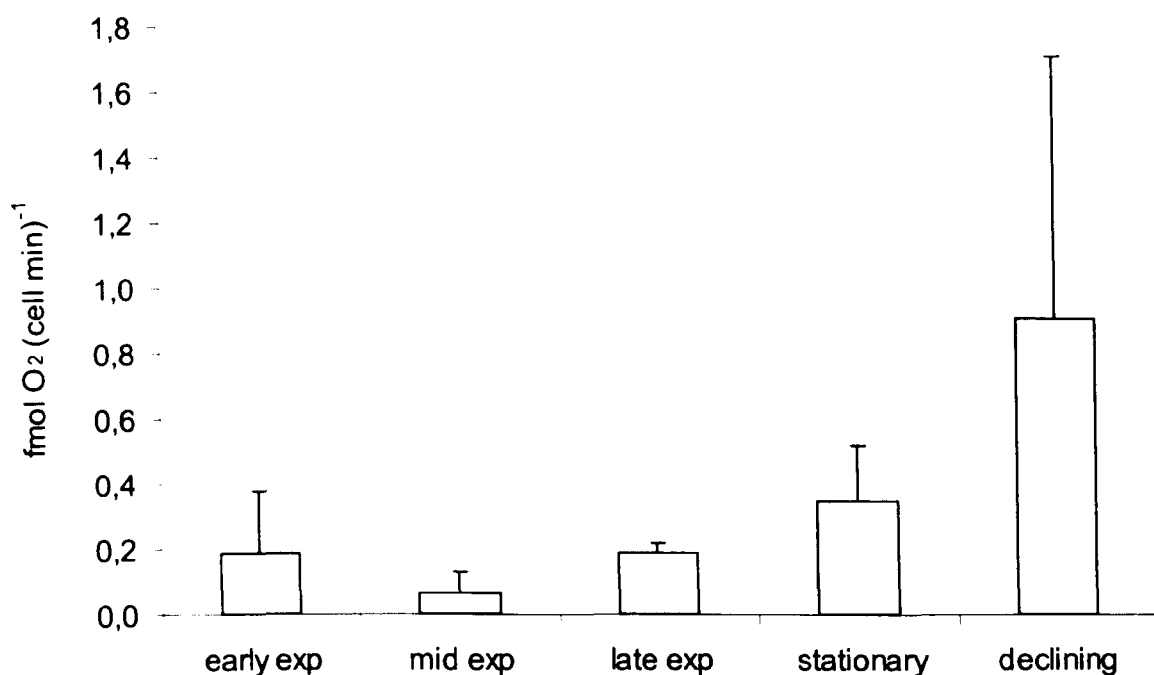


Figure 6-4: Mean oxygen consumption rate (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for protein [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] upon EPA-addition to cell lysate determined with the polarographic assay at pH=8.15 in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining).

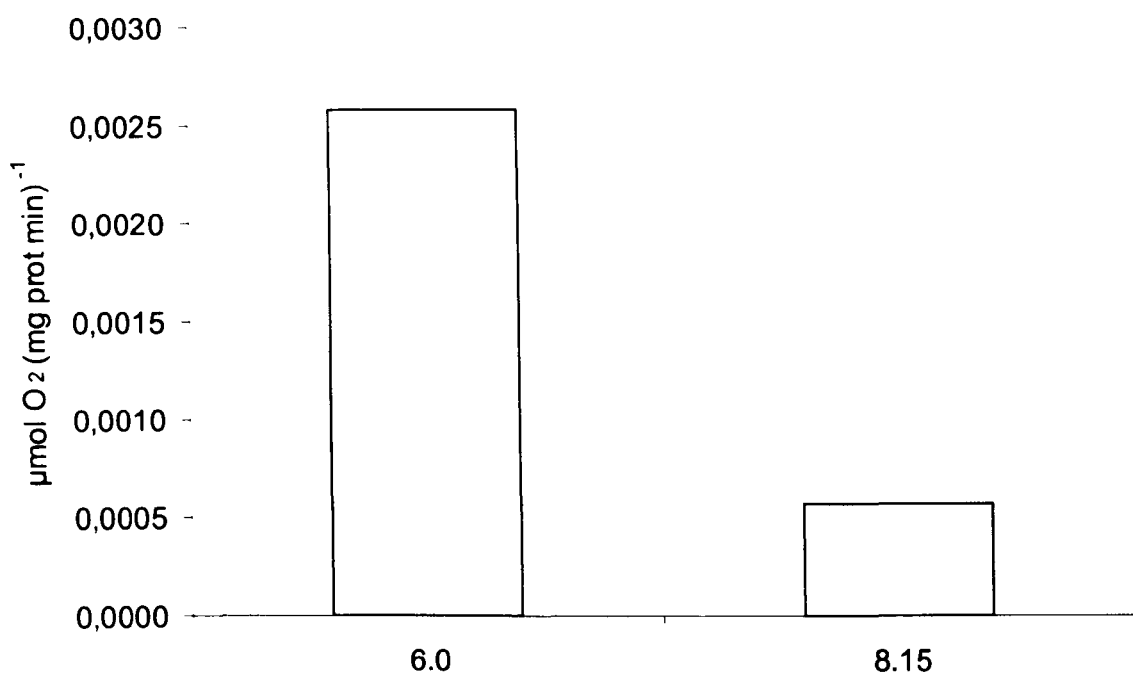


**Figure 6-5: Mean oxygen consumption rate (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for cells [fmol O<sub>2</sub> (cell min)<sup>-1</sup>] upon EPA-addition to cell lysate determined with the polarographic assay at pH=8.15 in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining).**

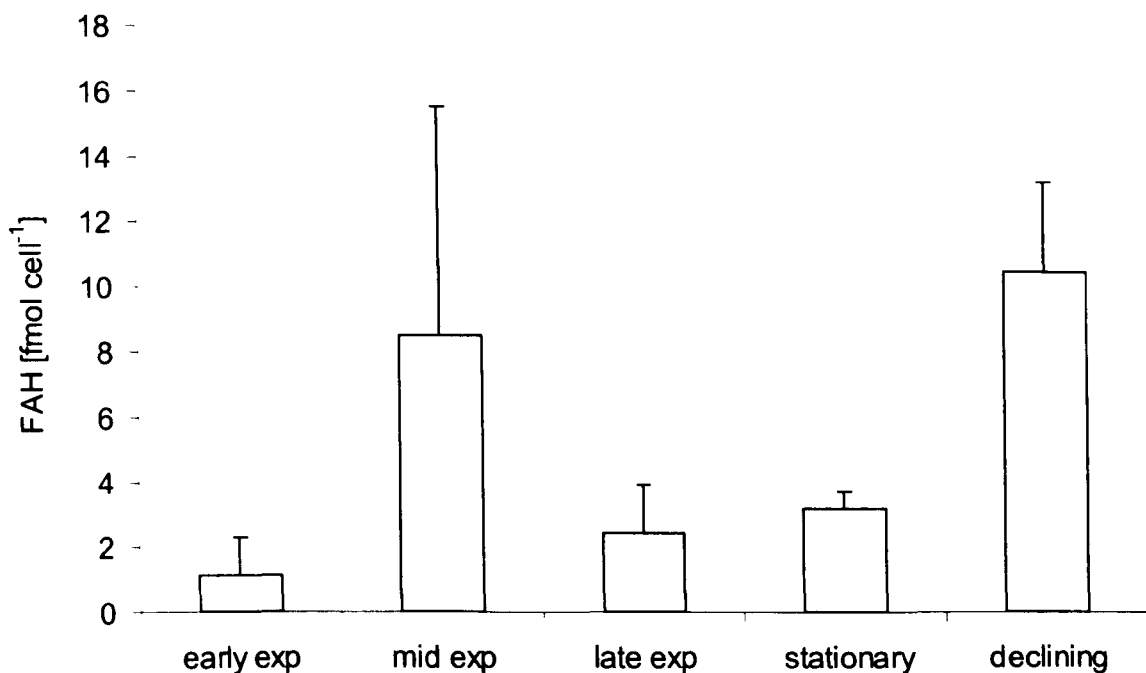
When oxygen consumption rate was normalized for cells, the values followed a similar pattern as cellular oxylipin production (Figure 6-2) with highest values ( $0.9 \pm 0.8$  fmol O<sub>2</sub> (cell min)<sup>-1</sup>) apparently found in the declining phase (Figure 6-5). Differences between phases were not statistically significant (one-way ANOVA:  $p > 0.05$ ).

#### 6.3.3.2 Colorimetric Assay

That lipoxygenase (LOX) enzymes were active at pH=6.0 in *Pseudo-nitzschia delicatissima* was indicated polarographically (Figure 6-6). In fact, the increase in oxygen consumption rate upon EPA-addition seemed greater at pH=6.0 than 8.15, suggesting that LOX enzymes in *P. delicatissima* may be more active at lower pH.

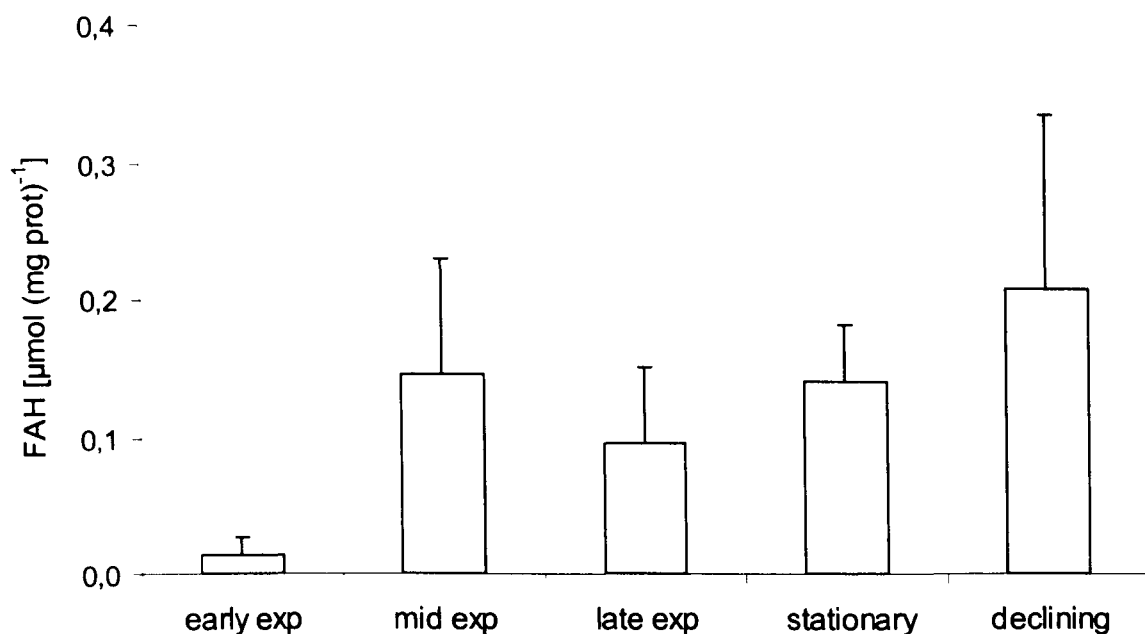


**Figure 6-6: Oxygen consumption rate normalized for protein  $[\mu\text{mol O}_2 (\text{mg prot min})^{-1}]$  upon EPA-addition to cell lysate (n=1) determined with the polarographic assay in *Pseudo-nitzschia delicatissima* at pH=6.0 and 8.15.**



**Figure 6-7: Mean fatty acid hydroperoxide (FAH) production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for cells [fmol cell<sup>-1</sup>] determined with the colorimetric assay in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining). FAH production was defined as the difference in FAH concentrations between cell lysates after 20 min and blanks.**

Therefore, the colorimetric assay was applied to the determination of fatty acid hydroperoxide (FAH) production in *P. delicatissima*. Also in this case, peak values were indicated for the declining phase when FAH production was normalized for cells (Figure 6-7). Differences between phases, however, were not statistically significant (one-way ANOVA:  $p > 0.05$ ).



**Figure 6-8: Mean fatty acid hydroperoxide (FAH) production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for protein [ $\mu\text{mol (mg prot)}^{-1}$ ] determined with the colorimetric assay in different growth phases of *Pseudo-nitzschia delicatissima* (early exponential, mid exponential, late exponential, stationary, and declining). FAH production was defined as the difference in FAH concentrations between cell lysates after 20 min and blanks.**

The pattern was less clear when FAH production was normalized for protein with similar FAH production in all phases (one-way ANOVA:  $p>0.05$ ) (Figure 6-8).

### 6.3.4 Oxylipin Composition

The three oxylipin compounds showed differential expression during the different growth phases (two-way ANOVA: interaction:  $p>0.05$ , oxylipin:  $p=0.0006$ , phase:  $p=0.03$ ) (Figure 6-9). The oxo-acid was present in low amounts (2.2-7.5 fg cell<sup>-1</sup>) and could be found only from the late exponential phase to the declining phase. The epoxyalcohol (13,14-HEpETE) and the hydroxy-acid (15S-HEPE) were continuously produced by the



culture during the entire growth curve, remaining at constant similar values up to the stationary phase ( $0.06\pm0.02$  and  $0.07\pm0.02$  pg cell<sup>-1</sup>, respectively). The amount of 15S-HEPE apparently increased during the declining phase up to  $0.31\pm0.31$  pg cell<sup>-1</sup>, even though this increase was not statistically significant (one-way ANOVA:  $p>0.05$ ). Production of 13,14-HEpETE reached its peak ( $0.23\pm0.13$  pg cell<sup>-1</sup>) in the stationary phase, which was a significant difference to the production in the early and mid exponential phases (one-way ANOVA:  $p=0.011$  and Tukey's post-test:  $p<0.05$ ).

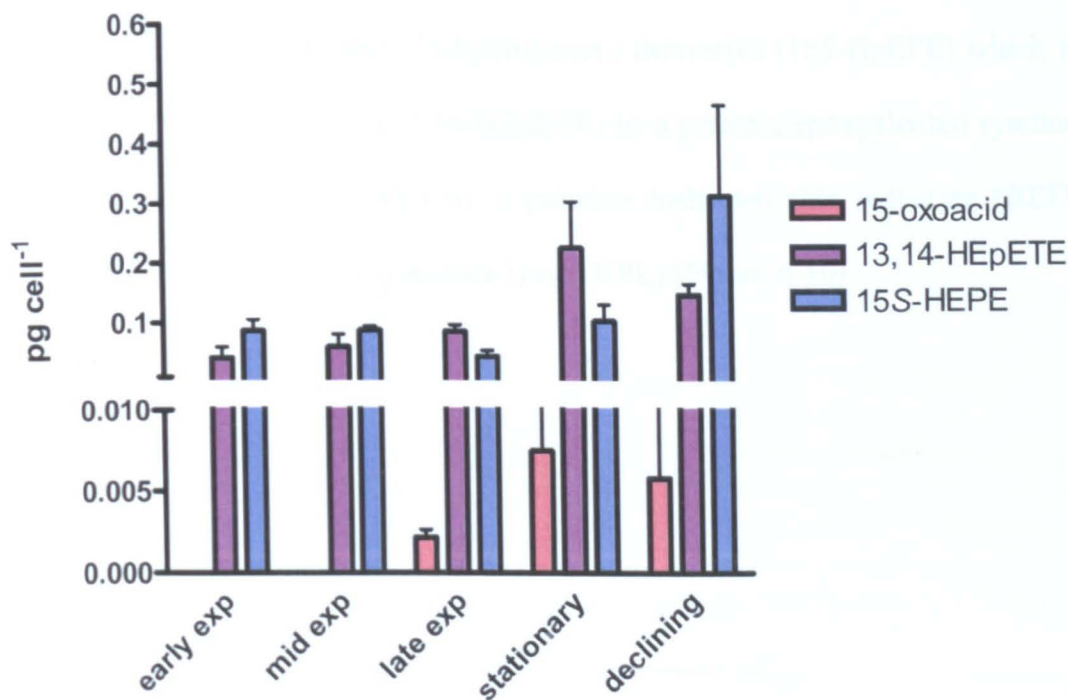
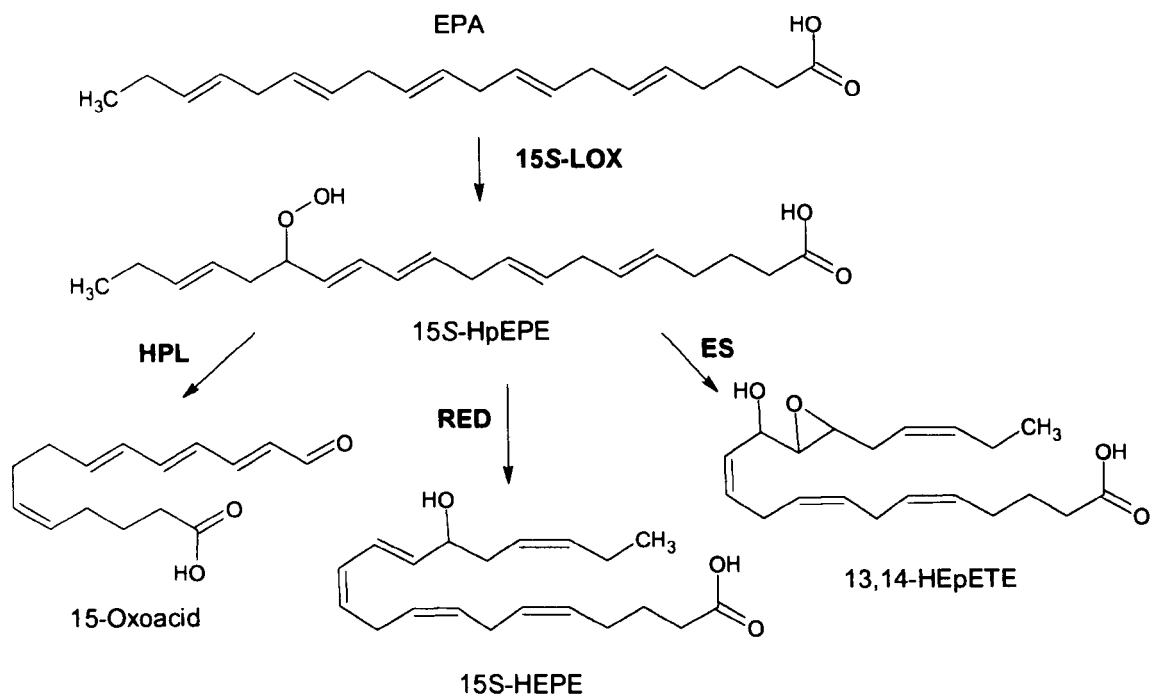


Figure 6-9: Mean production (+SEM; n=3 except early exp: n=5, declining: n=4) normalized for cells [pg cell<sup>-1</sup>] of the oxo-acid (15-oxoacid), the epoxyalcohol (13,14-HEpETE) and the hydroxy-acid (15S-HEPE) in different growth phases of *Pseudo-nitzschia delicatissima*.

## 6.4 Discussion

*Pseudo-nitzschia delicatissima* produced three major oxylipins deriving from lipoxygenase (LOX) activity on the C<sub>20</sub>-fatty acid eicosapentaenoic acid (EPA). These were the hydroxy-acid 15(*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*Z*,17*Z*)-eicosapentaenoic acid (15*S*-HEPE), the epoxyalcohol 13,14-*threo*-13*S*-hydroxy-14*S*,15*S*-*trans*-epoxyeicosa-5*Z*,8*Z*,11*Z*,17*Z*-tetraenoic acid (13,14-HEpETE) and 15-oxo-5*Z*,9*E*,11*E*,13*E*-pentadecatetraenoic acid. The first two oxylipins had been previously described in *Skeletonema marinoi* (Fontana *et al.* 2007b), whereas the oxo-acid was a novel oxylipin. All three oxylipins derive from the oxidation of EPA according to the followed proposed mechanism (Figure 6-10). EPA is oxidized by a 15*S*-LOX to the 15*S*-hydroperoxy derivative (15*S*-HpEPE) which is either converted to the epoxyalcohol (13,14-HEpETE) by a putative epoxyalcohol synthase (ES), to the hydroxy-acid (15*S*-HEPE) by a putative hydroperoxide reductase (RED) or to 15-oxoacid by a putative hydroperoxide lyase (HPL) (Figure 6-10).



**Figure 6-10: The proposed 15S-lipoxygenase (LOX) pathway in *Pseudo-nitzschia delicatissima*.** (EPA: Eicosapentaenoic acid, 15S-HpEPE: 15S-hydroperoxy eicosapentaenoic acid, HPL: hydroperoxide lyase, RED: hydroperoxide reductase, 15S-HEPE: hydroxy-eicosapentaenoic acid, ES: epoxyalcohol synthase, 13,14-HEpETE: epoxyalcohol).

No volatile aldehydes were detected in this diatom species in the present work. The aldehydes previously described by Miralto *et al.* (1999) in this species may have been an erroneous identification of the novel oxo-acid due to a similar chemical signal in NMR-analysis (A. Ianora, pers. comm.). In general, the production of oxylipins in this diatom increased sharply with the onset of stationary phase. This corresponded to an elevated LOX activity as shown by increased oxygen consumption rate upon EPA-addition to the cell lysate in the polarographic assay. Also the production of intermediate fatty acid hydroperoxides (FAHs) increased towards the end of culture growth. All three parameters for determining oxylipin metabolism therefore coincided in demonstrating the intensification of fatty acid oxidation during the stationary phase of *P. delicatissima*. When oxylipin production was normalized for cells, higher values were observed in the declining

phase compared to the stationary phase. Instead, when values were normalized for protein, they were highest in the stationary phase. The discrepancy of normalizing for protein compared to cells may depend on the decrease in live cells in the declining phase. Enzymes of lysed cells which are no longer considered in the cell count may still be present and active in the surrounding culture medium, contributing to oxylipin production. Since culture samples were collected by centrifugation, oxylipins from the culture medium may precipitate together with the cells and contribute to overall oxylipin production by the centrifuged cells, thereby increasing the value calculated per cell. In either case, whether oxylipin production was normalized for cells or for protein, oxylipin production increased in the latter phases of culture growth.

Elevated production of oxylipins in later phases of culture growth may be due to increased nutrient stress at elevated cell concentrations or to other stress factors triggered by higher culture density. This phenomenon has been observed in *S. marinoi*, which produces greater amounts of aldehydes under nutrient-limiting conditions and upon entry into the stationary phase in culture (Ribalet *et al.* 2007b). However, considering that production of these secondary metabolites is clearly modulated along the different phases of growth, they may also possess a signalling function. It is possible that these molecules have a regulatory role (Vidoudez and Pohnert 2008) and instead of being consequences of the culture entering into stationary phase are actually triggers involved in the decline of the culture (Vardi *et al.* 2006). This indicates that oxylipins may act akin to quorum sensing molecules in bacteria (Falciatore and Bowler 2002), regulating unicellular behaviour in a coordinated fashion, possibly leading to a coordinated senescence of the culture.

Furthermore, it is important to note that the three oxylipin compounds were produced differentially. The oxo-acid was present only upon entry into the stationary phase.

Furthermore, production of the oxo-acid in the stationary and senescent phase of the culture was highly variable between replicate cultures in the same growth phase. This suggests that the production of this molecule was highly sensitive to the physiological state of the algal culture. Therefore the oxo-acid may act as a specific signal regulating culture growth. Alternatively, the shift from exponential growth to stationary phase may result from the general increase in oxylipins or be depended on a complex interaction of several molecules.

Comparing culture growth to the development of a bloom at sea, these metabolites may then also be important in nature for controlling bloom termination (Vardi *et al.* 2006; Vidoudez and Pohnert 2008). A general increase in oxylipin production or the production of specific molecules, not normally present during exponential growth, may regulate a shift in growth phases as well as induce the end of a bloom. However, little is yet known about the factors determining bloom termination (see Chapter 7) and further work needs to be done on the possible involvement of oxylipins or other secondary metabolites in this process and the possible environmental stimuli or internal factors triggering their production.

## 6.5 Conclusions

*Pseudo-nitzschia delicatissima* demonstrated an increase in oxylipin metabolism with the progression of the growth curve. An intensification of fatty acid oxidative metabolism in the latter phases of culture growth was demonstrated by increased production of three major oxylipins, 15(*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*Z*,17*Z*)-eicosapentaenoic acid (15*S*-HEPE), 13,14-*threo*-13*S*-hydroxy-14*S*,15*S*-*trans*-epoxyeicosa-5*Z*,8*Z*,11*Z*,17*Z*-tetraenoic acid (13,14-HEpETE) and 15-oxo-5*Z*,9*E*,11*E*,13*E*-pentadecatetraenoic acid. The novel oxo-acid described in *P. delicatissima* was produced only upon entry into the stationary phase, whereas the production of 15*S*-HEPE and 13,14-HEpETE was continuous, even though it increased in the stationary and declining phases. Increased oxylipin production was correlated to an increase in lipoxygenase activity, measured as enhanced oxygen consumption rate in stationary phase, as well as intensified production of intermediate fatty acid hydroperoxides. These findings may suggest a regulatory role for oxylipins in culture growth and senescence, which implies a possible role for oxylipins in regulating bloom dynamics at sea.

## **7 Modulated Lipoxygenase Activity during a Bloom of *Skeletonema marinoi* in a Mesocosm Experiment**

The aim of the present experiment was to induce a mono-specific bloom of *Skeletonema marinoi* in a mesocosm set-up by inoculating mesocosm bags with various concentrations of a laboratory culture of *S. marinoi* in addition to nutrients. For the two higher concentrations employed, a bloom was successfully created and its oxylipin metabolism was followed as well as its impact on copepod reproductive success. *S. marinoi* produced aldehydes as well as other oxylipins in all mesocosms examined and production peaked during the demise of the bloom when oxylipin production was normalized for cells. This indicates a possible role for oxylipins as bloom regulators as already proposed for the regulation of culture growth. There was, however, no strong effect of the diatom bloom on copepod reproductive success. Hatching success remained high and abnormal nauplii were not observed, although there was some indication of an early detrimental effect on copepod reproduction in the form of apoptotic nauplii. Since the bloom was brief, feeding on *S. marinoi* may have induced apoptosis, but the effect was not strong enough to lead to the production of abnormal nauplii or to a decrease in hatching success.

## 7.1 Introduction

In the spring of 2008 (April 15<sup>th</sup>-28<sup>th</sup>), phytoplankton samples were collected during the course of a mesocosm experiment. This experiment was carried out at the mesocosm facility of the University of Bergen, Norway under the supervision of Jens Nejstgaard as part of an integrated EUROCEANS project. The goal was to create a mono-specific bloom of *Skeletonema marinoi*, identify triggers for such and analyze the effects of this bloom on the marine ecosystem. Special emphasis was given to the role of chemical signalling in shaping trophic interactions. Phytoplankton samples were collected for this purpose, more specifically for analyzing changes in lipxygenase (LOX) activity and oxylipin production in the mesocosms. Of interest were variations between different mesocosm treatments and also changes within one treatment over time. The aim was to correlate these data to the hatching success of *Calanus finmarchicus* in the different treatments. Furthermore, variations of LOX activity and oxylipin production were of interest for understanding metabolic variations during the development and decline of a bloom. As described in the previous chapter, oxylipins have been suggested as potentially important factors in shaping and possibly even terminating diatom blooms, based on laboratory studies (Vardi *et al.* 2006; Vidoudez and Pohnert 2008). This hypothesis was therefore tested in a controlled field situation.

Mesocosm experiments allow carrying out controlled experiments in a semi-natural environment. One factor can be varied, while others are kept as close to natural conditions as possible. For example, nutrient- or CO<sub>2</sub>-concentrations (Egge *et al.* 2009) can be varied while light and temperature are kept at environmental conditions. Temperature in our set-up for example was kept at ambient conditions by emerging mesocosms in the surrounding seawater. Also light conditions were natural as the mesocosms were set up



outside and the employed polyethylene bags let pass ~90% of photosynthetic radiation. These semi-controlled experiments can be very useful in bridging laboratory studies and field observations. Especially when studying the toxic effect of diatoms, laboratory studies have been criticized for not being ecologically relevant (Jonasdottir *et al.* 1998). Although mesocosms are not equivalent to field studies, they still manage to provide a closer approximation to natural systems, increasing complexity. Still they allow the specific manipulation of one or multiple factors in a closed off environment to predict the possible effect of those factors e.g. of iron fertilization on phytoplankton growth (Takeda *et al.* 2000).

Mesocosms have become interesting for following the development and decline of phytoplankton blooms and identifying possible triggers for bloom development as well as reasons for bloom decline. Phytoplankton blooms in general are triggered by increasing surface irradiance and nutrient availability in spring (Arnone *et al.* 1993). In fact, phytoplankton blooms in mesocosms are usually triggered by the addition of nutrients (Egge *et al.* 2009), but also the role of irradiance has been studied (Agusti *et al.* 2009). More interesting are the factors for bloom decline. It is still not clear whether phytoplankton blooms end because of nutrient depletion (Saito *et al.* 2006), grazing pressure (Riebesell *et al.* 1995; Anderson and Rengefors 2006), bacterial or viral attack (Bratbak *et al.* 1993; Gastrich *et al.* 2004; Simis *et al.* 2005), cell-cell signalling (Vardi *et al.* 2006; d'Ippolito *et al.* 2009), aggregation and subsequent sinking of phytoplankton cells (Kiorboe *et al.* 1996; Boyd *et al.* 2005), physical advection (Tester and Steidinger 1997) or a complex interaction of several of these factors.

In this experiment, the goal was to create a homogeneous bloom of just one diatom species, *S. marinoi*. By controlling the nutrient load of the mesocosm, one can control

phytoplankton composition. For example, adding silica in addition to nitrate and phosphate results in a predominance of diatoms (Egge and Aksnes 1992; Egge and Jacobsen 1997; Sommer *et al.* 2004). To create a specific diatom bloom, apart from adding nutrients including silica, varying concentrations of *S. marinoi* were added in this experimental set-up. This way it was also hoped to gain further knowledge on the necessary starting conditions for creating a mono-specific bloom. In a pilot experiment it had been determined that an initial concentration of 1000 cells ml<sup>-1</sup> of *S. marinoi* resulted in a specific bloom of this alga, whereas a ten times lower concentration did not result in a mono-specific bloom. Therefore in this experiment an intermediate concentration of 400 cells ml<sup>-1</sup> was also tested. The following results will focus on oxylipin metabolism of *S. marinoi* during the bloom and on its effects on the secondary consumer *C. finmarchicus*. The possible role of oxylipins in bloom termination will also be discussed.

## 7.2 Materials and Methods

### 7.2.1 Experimental Set-up

Mesocosms consisted of polyethylene bags with a circumference of 1 m and a depth of 5 m for a total volume of 30 m<sup>3</sup>. Bags were suspended on a floating mesocosm facility immersing them in the surrounding seawater at about 200 meters from shore in a protected bay of the Raunefjord. Six different treatments were set up (Table 7-1). All bags were filled with post-bloom nutrient poor seawater pumped up from 40 m depth. A bubbling system guaranteed mixing of the water column. Bag A contained only seawater to control for a possible “bag effect”. It was assumed that this treatment would behave as the surrounding sea. Bag B was enriched with the macronutrients nitrate and phosphate. Here a

mixed phytoplankton bloom was expected. Bag C was additionally enriched in silicate to promote a mixed diatom bloom. In bags D, E, and F, additionally to nutrients, varying concentrations of *Skeletonema marinoi* were added to promote a mono-algal bloom of this species. Cell concentrations given for *S. marinoi* refer to the initial cell concentrations after inoculation (Table 7-1). All bags were inoculated with the same exponentially growing culture of the local strain of *S. marinoi* grown in bulk in the laboratory.

**Table 7-1: Mesocosm bag treatments (Bags A-F) (cell counts in D-F refer to the initial concentration of *Skeletonema marinoi* upon inoculation) and the parameters analyzed in the various treatments (oxylipin production and reproductive success).**

Bag	Treatment	Oxylipin production	Reproductive success
A	None		
B	+Nitrate + Phosphate		X
C	+Nitrate + Phosphate +Silicate	X	X
D	+Nutrients +100 cells ml <sup>-1</sup>		
E	+Nutrients +400 cells ml <sup>-1</sup>	X	
F	+Nutrients +1000 cells ml <sup>-1</sup>	X	X

### 7.2.2 Sample Analysis

Three of the treatments were analyzed for oxylipin production (Table 7-1), the treatment inoculated with just nutrients (mesocosm C), which was used as a control, and the two treatments inoculated with 400 and 1000 cells ml<sup>-1</sup> initial *S. marinoi* cell concentrations

(mesocosms E and F), in which blooms of *S. marinoi* were expected. These treatments were sampled on the following days after inoculation (Table 7-2).

**Table 7-2: Sampling days (days after inoculation) for analysis of oxylipin production in mesocosm bags C, E, and F.**

Day	4	5	6	7	8	9	10	11	12
C				X	X	X	X		X
E				X	X	X	X		X
F	X		X	X	X	X	X		X

7.2.2.1 *Sample Collection*

Phytoplankton samples were to be collected by filtering onto a submerged phytoplankton net (20 µm) to avoid damage to cells. This, however, was not successful because algae passed through the net. After trying several means of filtering phytoplankton samples directly onto filters, a method was established in which 4 x 100-250 ml were filtered separately onto 1.0-µm polycarbonate filters (GE Water & Process Technologies) with a vacuum pump for a total of 400-1000 ml filtered of mesocosms C, E, and F per sampling day. These filters were folded into Eppendorf tubes, frozen in liquid nitrogen and kept at -80°C until shipping to SZN in dry ice.

#### 7.2.2.2 Method Development

Since samples were usually analyzed as diatom pellets, methodological tries were carried out to determine how to best analyze filters, especially as sample amounts on filters were low, only  $83 \pm 72$  mg corresponding to about a million cells of *Skeletonema marinoi*. To estimate how many of the four filters needed to be combined for the different analyses, a culture of the *Skeletonema* strain used in the mesocosm experiment and grown at SZN was filtered onto the same filters used during the mesocosm experiment (1.0- $\mu$ m polycarbonate). 100 ml of culture was filtered onto each filter, which corresponds to about 20 mg of sample or  $4.7 \cdot 10^6$  cells. The discrepancy in filter weights compared to cell amounts between the mesocosm samples and the culture samples was due to the fact that the latter filters consisted of a pure *Skeletonema* culture, whereas mesocosm samples were mixed phytoplankton samples. Filters were folded into Eppendorf tubes and frozen, analogous to mesocosm samples prior to analysis.

The usual analysis consisted of suspending diatom pellets in 1 ml  $\text{H}_2\text{O}_{\text{deionised}}$  per g sample (2.2.2.2). Filters in Eppendorf tubes were suspended in 1 ml  $\text{H}_2\text{O}_{\text{deionised}}$  (Milli-Q), which was the minimum amount required for sonicating and handling of the sample. This, however, resulted in a dilution of 1:50 compared to standard procedure (20 mg in 1 ml instead of 1 g). The sample was sonicated (2\*30 s on ice at 20% output, Branson sonifier 250) with the filter still inside the Eppendorf tube, because filters were not damaged by sonication. This way as many cells as possible were removed from the filters before removing them with tweezers and rinsing them several times with the cell lysate. Filters checked under the microscope resulted relatively free of cell residue.

Analogous to other culture studies, adding 200  $\mu$ l of cell lysate corresponding to about 4 mg sample in the colorimetric assay gave a good reading of absorbance in the

spectrophotometer (Table 7-3). Values were readily distinguishable from the solution blank.

**Table 7-3: Absorbance at 598 nm of *Skeletonema marinoi* cell lysate corresponding to varying amounts of pellet wet weight [mg] in the colorimetric assay.**

Pellet wet weight [mg]	Absorbance (598 nm)
Solution blank	0.013
0.4	0.020
0.8	0.026
4.0	0.126

Oxygraph measurements with one of these filters showed rapid oxygen consumption of the cell lysate, especially considering the very low sample amount added. However, the increase in oxygen consumption rate upon the addition of eicosapentaenoic acid (EPA) to the cell lysate was minimal (Table 7-4). Possibly, the cell lysate was consuming oxygen in part due to lipoxygenase (LOX) activity, but was not using exogenous EPA.

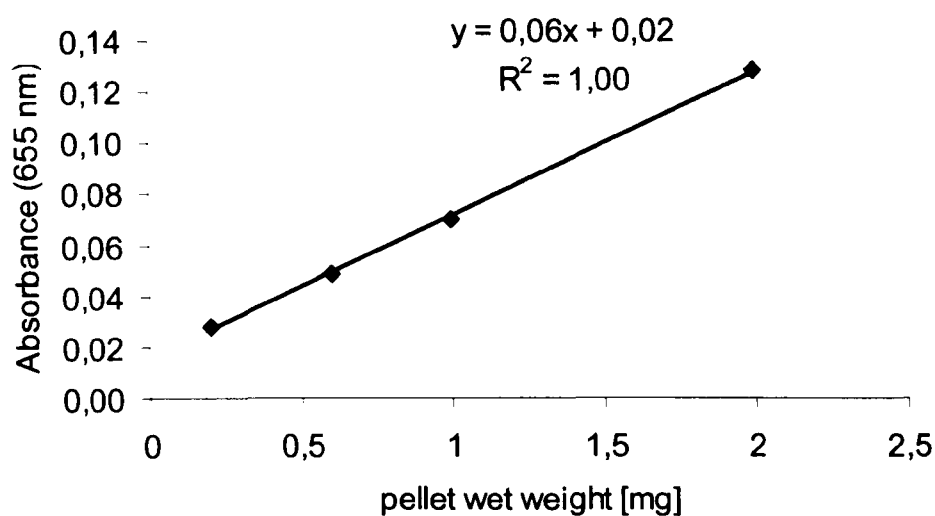
**Table 7-4: Oxygen consumption after 5 min [μmol] in the polarographic assay at pH=8.15 in *Skeletonema marinoi* cell lysate corresponding to varying amounts of pellet wet weight [mg] incubated without (lysate) and with eicosapentaenoic acid (lysate+EPA).**

μmol O <sub>2</sub> consumed after 5 min		
Blank	0.002	
Pellet wet weight [mg]	Lysate	Lysate+EPA
0.005	0.009	0.012
0.010	0.024	0.026

For protein analysis, samples were usually diluted 1:5 for a concentration of 0.2 mg μl<sup>-1</sup> sample solution. By not diluting further, concentrations in the present samples were ten times lower at 0.02 mg μl<sup>-1</sup>, which gave a satisfactory reading with aliquots of 10-100 μl (0.2-2.0 mg sample wet weight) in the assay for protein determination (Table 7-5). Absorbance values were linear to the amount of pellet wet weight added, even though the relationship was not 1:1 (Figure 7-1). However, since protein content was calculated via a standard curve carried out with BSA (2.2.6), this was of little importance.

**Table 7-5: Absorbance at 655 nm of *Skeletonema marinoi* cell lysate corresponding to varying amounts of pellet wet weight [mg] in the assay for protein determination.**

Pellet wet weight [mg]	Absorbance (655 nm)	Absorbance (Sample-Blank)
Blank	0.21	
0.2	0.24	0.03
0.6	0.26	0.05
1.0	0.28	0.07
2.0	0.34	0.13



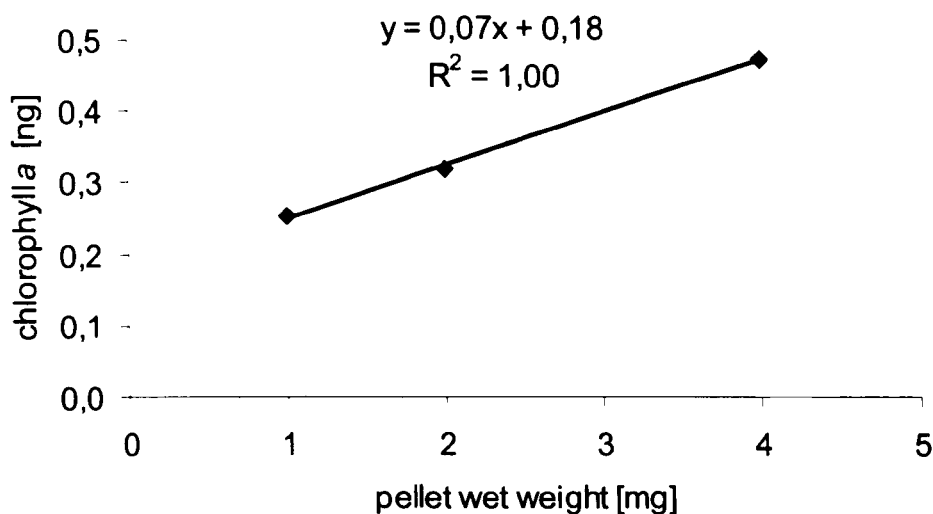
**Figure 7-1: Linear relationship of absorbance of cell lysate (655 nm) to *Skeletonema marinoi* pellet wet weight [mg] in the assay for protein determination.**

Chlorophyll measurements were carried out directly on a sample from the mesocosm and turned out to be more problematic. Although chlorophyll *a* amount determined was linear to the amount of sample added, there was no 1:1 relationship (Table 7-6, Figure 7-2). Since no standard curve was available for chlorophyll measurements, chlorophyll determination was abandoned for mesocosm samples.

**Table 7-6: Chlorophyll *a* [ng] determined in *Skeletonema marinoi* cell lysate, depending on pellet wet weight used in the assay [mg].**

Pellet wet weight [mg]	Chlorophyll <i>a</i> [ng]
1.0	0.25
2.0	0.32
4.0	0.47





**Figure 7-2: Linear relationship of chlorophyll *a* amount [ng] to various amounts of *Skeletonema marinoi* pellet wet weight [mg].**

Extraction of the trial filters for aldehyde analysis (two replicates) showed no detectable aldehydes, whereas oxylipins other than aldehydes could be detected without difficulties and values calculated per cell were quite high (4.5 pg cell<sup>-1</sup>). Assuming that the lack of detectable aldehydes may be due to low sample amounts, all four filters collected from each mesocosm per day were pooled and extracted for quantification of oxylipin production, including subsampling for protein. For calculation purposes, chlorophyll *a* values measured independently in the mesocosms were used. Although detection of LOX activity was feasible for the filter samples, analysis of aldehydes and other oxylipins was judged more important and sample amount was not sufficient to allow also the measurement of LOX activity.

### 7.2.3 Culture Samples

The strain of *Skeletonema marinoi* used for inoculation of the mesocosms had been originally isolated from the Bergen fjord. A culture was brought back from Bergen and cultured at SZN, keeping culture conditions as similar as possible. Cultures were kept in a climate chamber on a 12h:12h light:dark cycle ( $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) analogous to the Adriatic *S. marinoi*, but at a temperature of 10°C.

Two culture samples of *S. marinoi* were collected in Bergen, one from the actual culture used for inoculation of the mesocosms, which was in exponential phase at the time of sampling and the other from a culture grown in parallel, which was in stationary phase at the time of sampling. Both samples were collected by centrifugation, frozen and analyzed at SZN together with two samples from the culture grown directly at SZN. Culture samples were analyzed for lipoxygenase (LOX) activity and oxylipin production as described in Chapters 2 (2.2.3, 2.2.4) and 3 (3.2.2).

### 7.2.4 Copepod Reproduction

Copepod incubation experiments to determine daily egg production rates and egg viability were carried out for mesocosms B (+N, +P), C (+N, +P, +Si), and F (+N, +P, +Si, +*Skeletonema*) (Table 7-1). *Calanus finmarchicus* females were sorted from zooplankton samples collected from the fjord. 15 females for each mesocosm treatment were incubated individually in 50-ml Falcon culture flasks filled with water from the respective mesocosms. Flasks were kept in a temperature-controlled room at in situ temperature (10°C) at dim light. Females were transferred daily with a wide-mouthed glass pipette into new flasks with fresh mesocosm water from that day. The water sample from the previous

day was gently poured into 100-ml crystallizing dishes and eggs as well as faecal pellets produced in 24 h enumerated under an inverted microscope (2.5x) in the temperature-controlled room. After counting, the sample was poured gently back into the flask and eggs left to hatch for 72 h in the temperature-controlled room after which they were fixed with buffered formalin (4% final concentration) before counting the number of hatched nauplii, abnormal nauplii, and non-viable eggs. Nauplii were analyzed for apoptosis by the TUNEL-staining kit (Roche) after fixation in 1% PBS and 0.02% sodium azide.

### **7.2.5 Data Analysis**

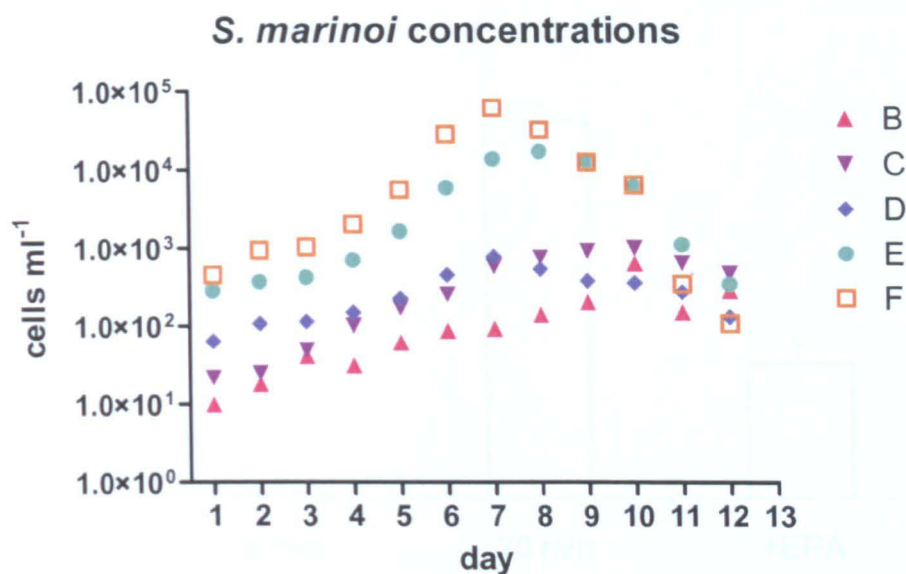
Oxylipin production was normalized for cells to observe changes in cellular oxylipin production during the course of the bloom. However, to have an idea of the possible effect of oxylipins on the zooplankton population, the contribution of oxylipin-producing *Skeletonema marinoi* to the total phytoplankton biomass was also taken into account. To this end, oxylipin production was normalized for chlorophyll *a* and carbon determined independently in the mesocosm samples and compared to reproductive success of copepods.

## 7.3 Results

### 7.3.1 Mesocosm Samples

#### 7.3.1.1 Bloom Development

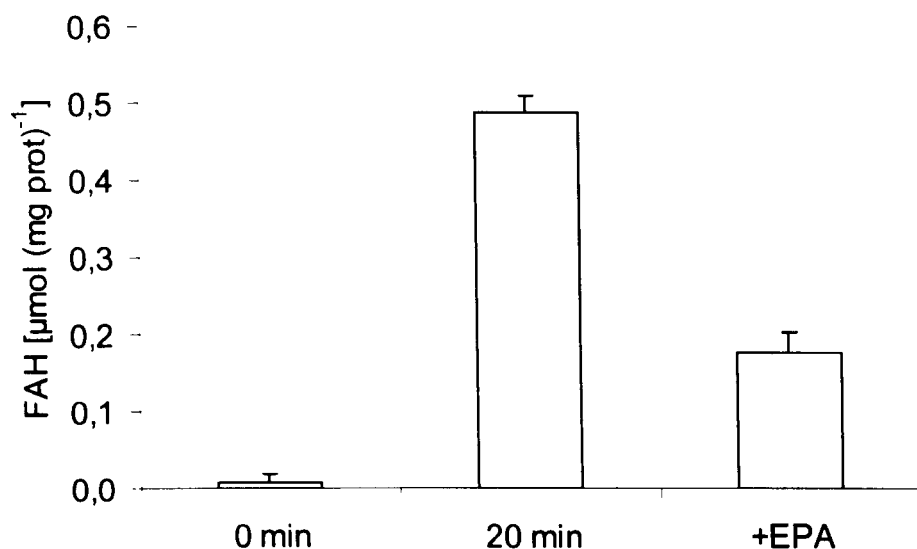
A bloom of *Skeletonema marinoi* developed in both mesocosms inoculated with higher initial concentrations of this alga (mesocosms E and F) (Table 7-1, Figure 7-3). No bloom of *S. marinoi* developed in the mesocosm inoculated with the lowest concentration of this alga (mesocosm D). Peak cell concentrations were reached on day 7 in mesocosm F (60,000 cells ml<sup>-1</sup>) and on day 8 in mesocosm E (17,000 cells ml<sup>-1</sup>). Higher maximum cell concentrations were reached in mesocosm F, which had been inoculated with higher initial cell concentrations (a calculated value of 1000 compared to 400 cells ml<sup>-1</sup>) (Table 7-1). Also in mesocosms B and C, *Skeletonema* abundance increased slightly during the course of the experiment. However cell concentrations remained low at ~600 and ~1000 cells ml<sup>-1</sup> in mesocosms B and C, respectively.



**Figure 7-3:** Cell concentrations [cells ml<sup>-1</sup>] of *Skeletonema marinoi* in mesocosms B, C, D, E, and F during the course of the mesocosm experiment (days after inoculation).

### 7.3.1.2 Lipxygenase Activity

Only one sample from the mesocosm was analyzed for lipxygenase (LOX) activity in the colorimetric assay (mesocosm F, day 10) (Figure 7-4).

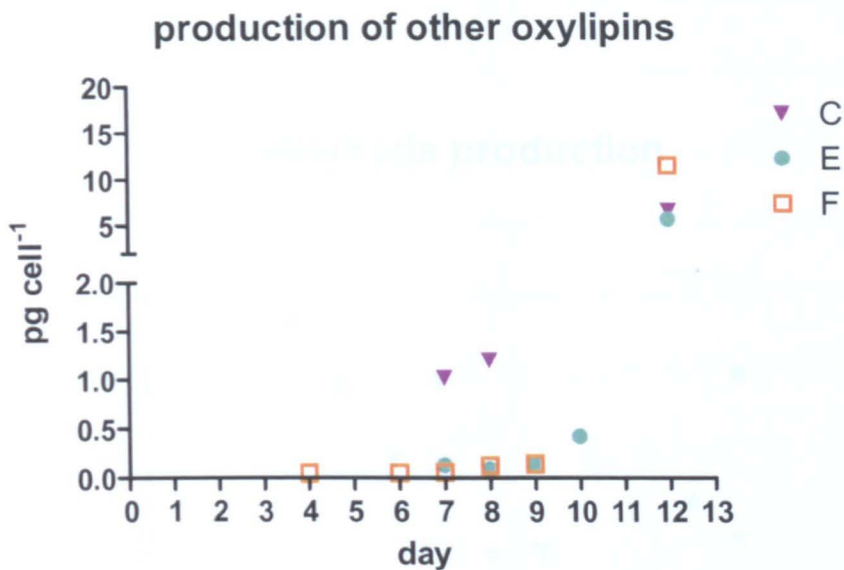


**Figure 7-4: Mean concentrations (+SEM, n=2) of fatty acid hydroperoxide (FAH) normalized for protein [ $\mu\text{mol (mg prot)}^{-1}$ ] determined with the colorimetric assay in blanks (0 min), in cell lysate of a mesocosm sample (mesocosm F, day 10) (20 min), and in cell lysate upon EPA-addition (+EPA).**

This sample collected from the declining phase of the bloom showed an apparently strong increase in fatty acid hydroperoxide (FAH) concentration from the blank (0 min) to the sample (20 min) (Figure 7-4). Upon addition of EPA, there seemed to be a decrease in FAHs.

### 7.3.1.3 Oxylipin Production

Except for in one sample (mesocosm F, day 6), which corresponded to the day before the peak of the bloom in the mesocosm with highest *Skeletonema* concentrations, no aldehydes were detectable in the samples by the extraction method used. Particulate and dissolved aldehydes were, however, detected by a different method in this experiment (Vidoudez, in preparation) and these data were used for comparative purposes (Figure 7-6).



**Figure 7-5: Production of oxylipins other than aldehydes (n=1) normalized for cells [pg cell<sup>-1</sup>] in mesocosm bags C, E, and F during the course of the experiment (days after inoculation).**

When oxylipins other than aldehydes were normalized for cells, values were relatively low during the entire experiment and only increased towards the end (Figure 7-5). This trend was observed in all three mesocosms examined for oxylipin production (C, E, and F) with maximum values of 6.5, 5.7, and 11.5 pg cell<sup>-1</sup> respectively, reached on day 12 after inoculation. In mesocosm C, which had low cell concentrations of *Skeletonema marinoi*, oxylipin values reached a similar peak production as in mesocosm E and there were no significant differences between the treatments (repeated measures ANOVA:  $p>0.05$ ).

As aldehyde data were available as molar concentrations, data for oxylipins other than aldehydes were also converted to molar concentrations to allow for comparisons (Figure 7-6).

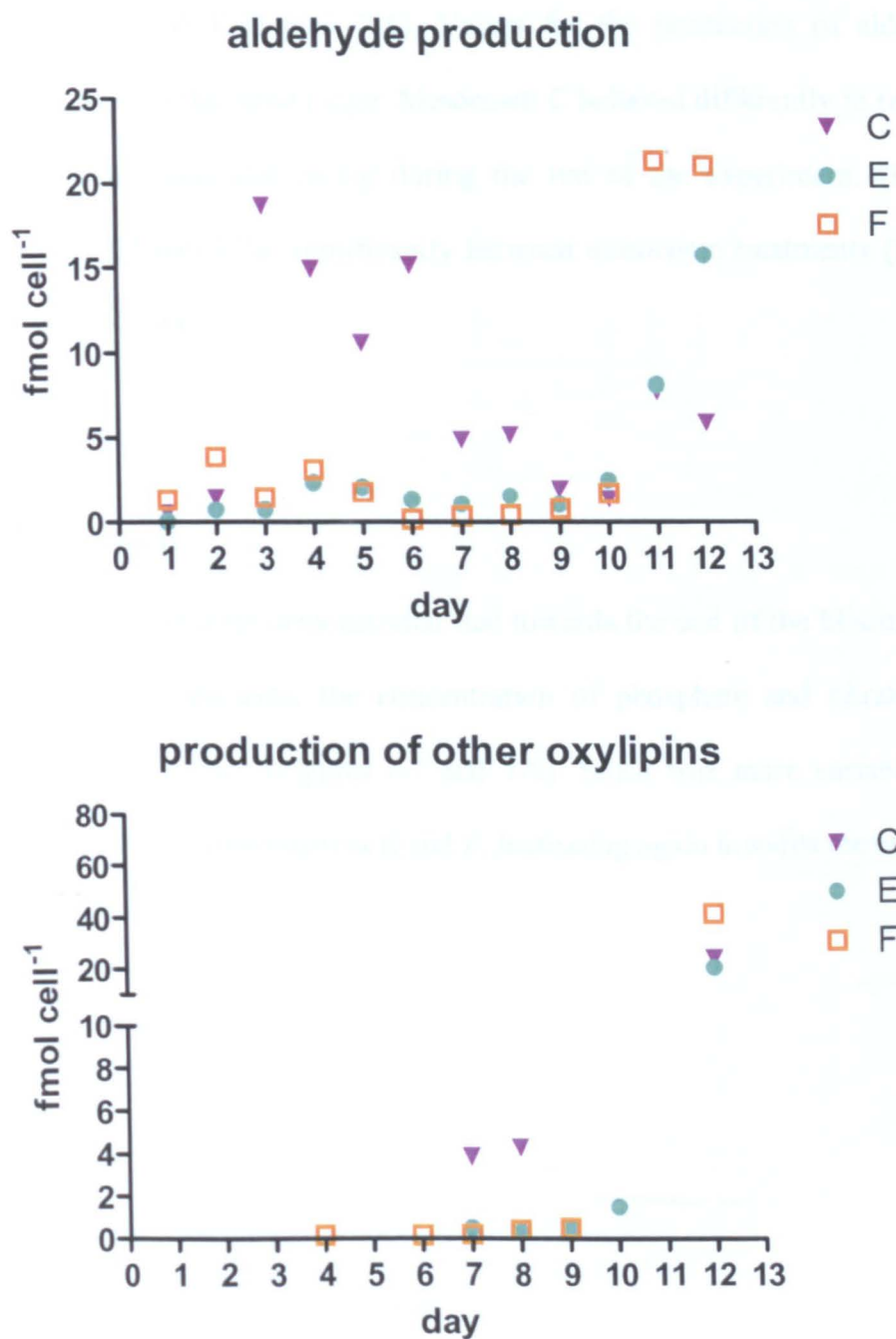


Figure 7-6: Production of aldehydes and oxylipins other than aldehydes (n=1) normalized for cells [fmol cell<sup>-1</sup>] in mesocosm bags C, E, and F during the course of the experiment (days after inoculation).



A similar trend of cellular production was observed for aldehydes and other oxylipins. Also the cellular production of aldehydes increased towards the end of the bloom in mesocosms E and F (Figure 7-6). Values for the production of aldehydes and other oxylipins were in the same range. Mesocosm C behaved differently in regard to aldehydes with per cell values decreasing during the run of the experiment. However, aldehyde production did not differ significantly between mesocosm treatments (repeated measures ANOVA:  $p>0.05$ ).

### **7.3.2 Nutrients**

Nutrient measurements demonstrated that towards the end of the bloom in all mesocosms inoculated with nutrients, the concentration of phosphate and nitrate had returned to pre-inoculation values (Figures 7-7 and 7-8). Silica was more variable and was lowest during the bloom in mesocosms E and F, increasing again towards the end (Figure 7-9).

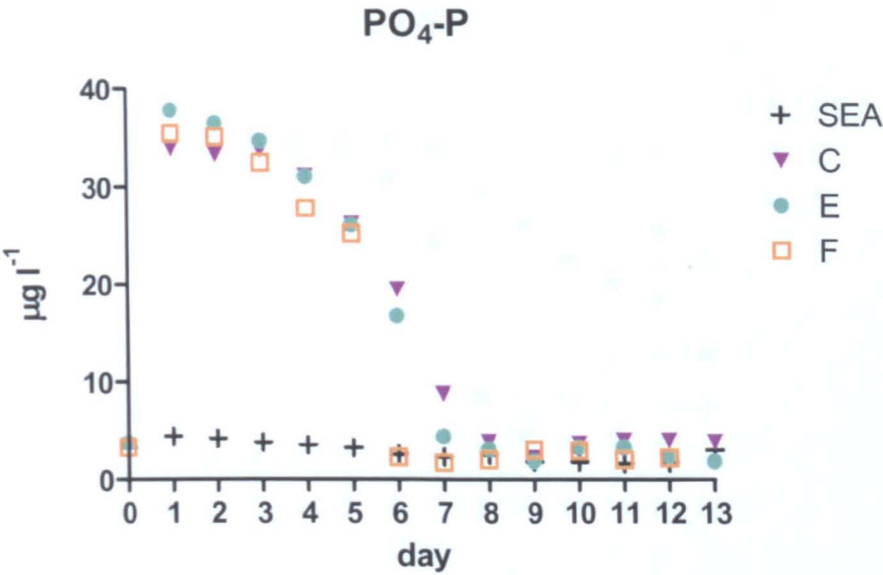


Figure 7-7: Concentration of phosphate [ $\mu\text{g l}^{-1}$ ] ( $n=1$ ) in mesocosm bags C, E, and F during the course of the experiment (days after inoculation; surrounding sea measured as control).

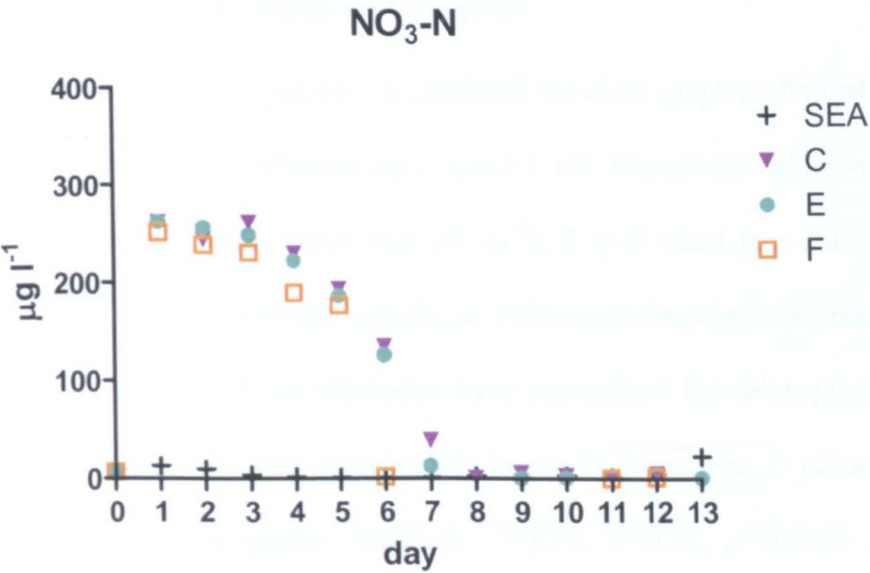


Figure 7-8: Concentration of nitrate [ $\mu\text{g l}^{-1}$ ] ( $n=1$ ) in mesocosm bags C, E, and F during the course of the experiment (days after inoculation; surrounding sea measured as control).

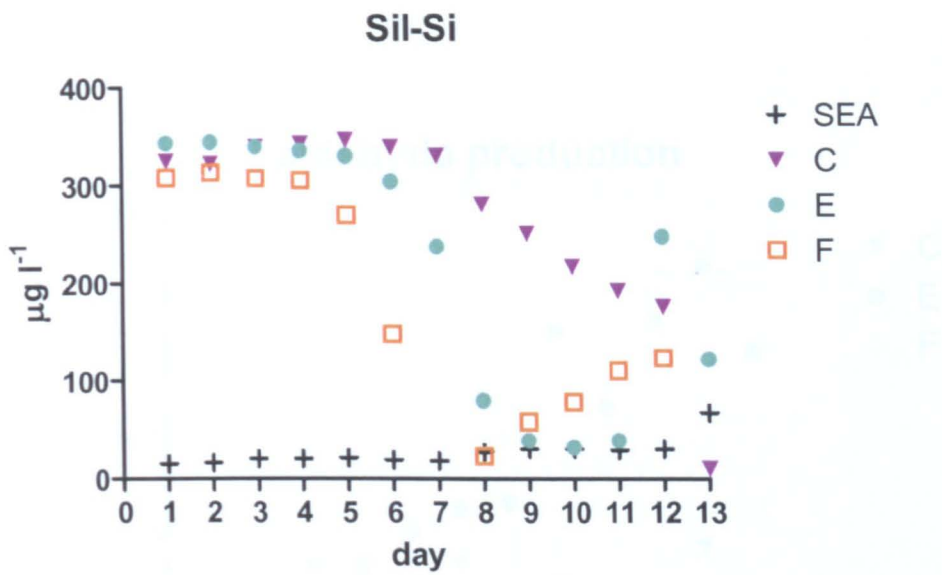


Figure 7-9: Concentration of silicate [ $\mu\text{g l}^{-1}$ ] ( $n=1$ ) in mesocosm bags C, E, and F during the course of the experiment (days after inoculation; surrounding sea measured as control).

### 7.3.3 Effects of Oxylipins on Copepods

Oxylipins other than aldehydes normalized for chlorophyll *a* showed a continuous increase towards the end of the experiment (Figure 7-10). Maximum values were reached on day 12 in mesocosms C ( $0.91 \text{ nmol } (\mu\text{g chl } a)^{-1}$ ), E ( $1.8 \text{ nmol } (\mu\text{g chl } a)^{-1}$ ), and F ( $1.2 \text{ nmol } (\mu\text{g chl } a)^{-1}$ ). There were no significant differences between treatments (repeated measures ANOVA:  $p>0.05$ ). When aldehydes were normalized for chlorophyll *a*, on the other hand, aldehyde production was significantly lower in mesocosm C compared to mesocosms E and F (repeated measures ANOVA:  $F=9.75$ ,  $r^2=0.47$ ,  $p=0.0009$  and Tukey's posttest:  $p<0.01$ ).

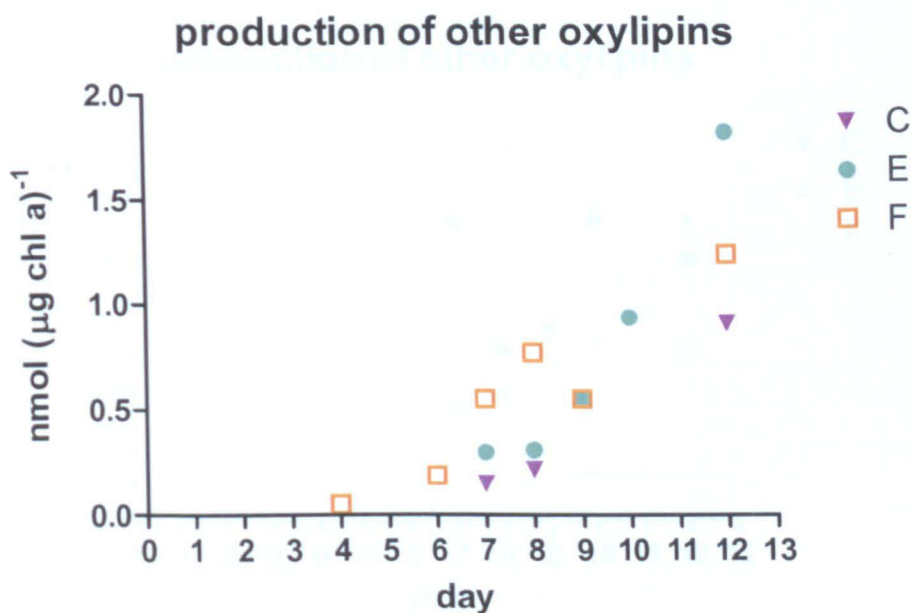
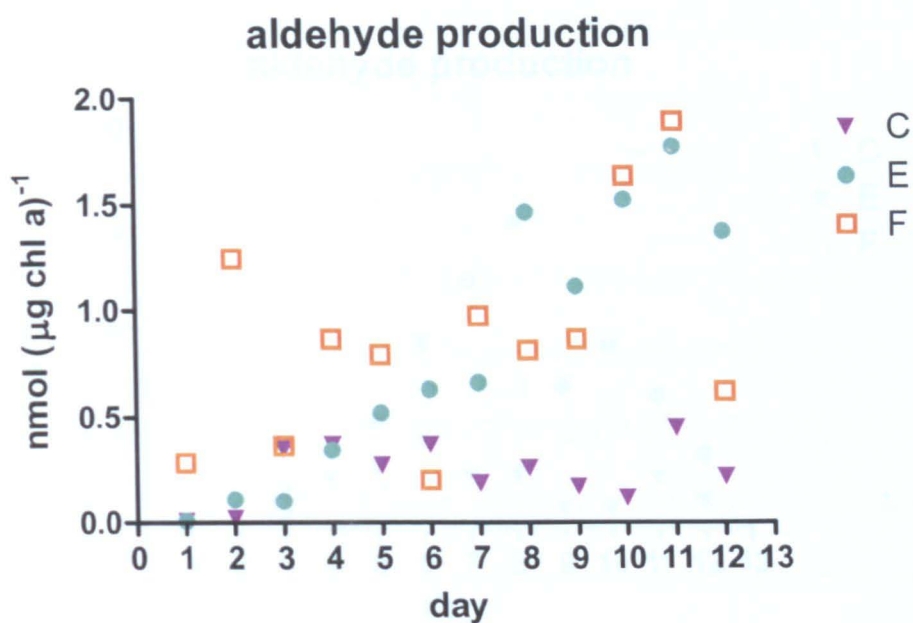


Figure 7-10: Production of aldehydes and oxylipins other than aldehydes (n=1) normalized for chlorophyll *a* [nmol ( $\mu\text{g chl } a$ )<sup>-1</sup>] in mesocosm bags C, E, and F during the course of the experiment (days after inoculation).

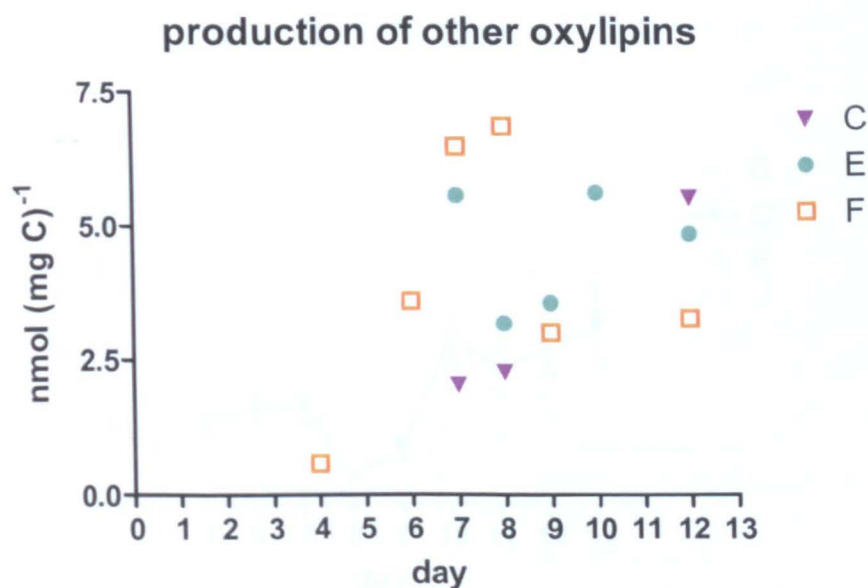
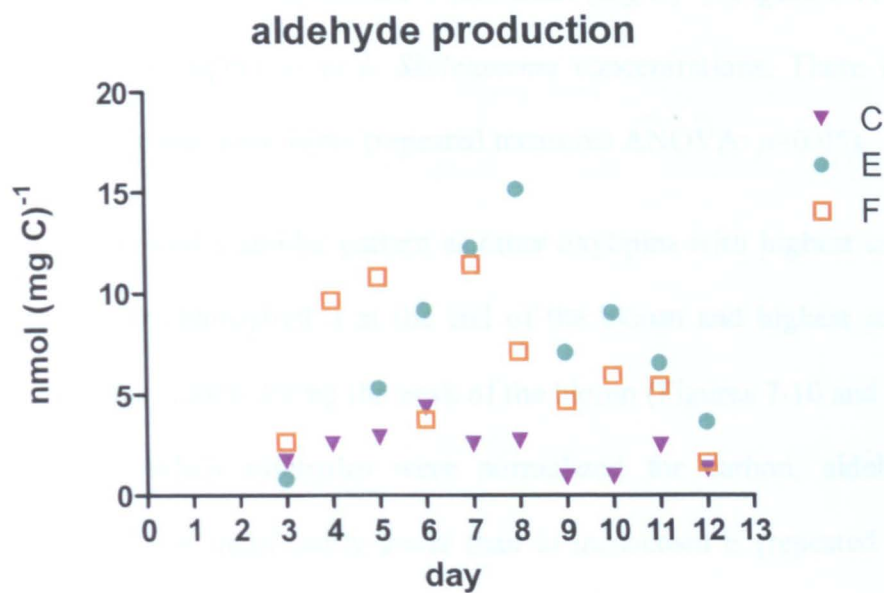


Figure 7-11: Production of aldehydes and oxylipins other than aldehydes (n=1) normalized for carbon [nmol (mg C)<sup>-1</sup>] in mesocosm bags C, E, and F during the course of the experiment (days after inoculation).

When oxylipins other than aldehydes were normalized for carbon, peak concentrations were found slightly after the peak of the bloom, on day 8 in mesocosm F (6.9 nmol (mg C)<sup>-1</sup>) and day 10 in mesocosm E (5.6 nmol (mg C)<sup>-1</sup>) (Figure 7-11). Maximum values were shifted in respect to peak *Skeletonema* concentrations. There were no significant differences between treatments (repeated measures ANOVA:  $p>0.05$ ).

Aldehydes showed a similar pattern as other oxylipins with highest concentrations, when normalized for chlorophyll *a* at the end of the bloom and highest concentrations, when normalized for carbon during the peak of the bloom (Figures 7-10 and 7-11) (Vidoudez, in preparation). When aldehydes were normalized for carbon, aldehyde production in mesocosm C was significantly lower than in mesocosm E (repeated measures ANOVA:  $F=4.64$ ,  $r^2=0.34$ ,  $p=0.024$  and Tukey's posttest:  $p<0.05$ ).

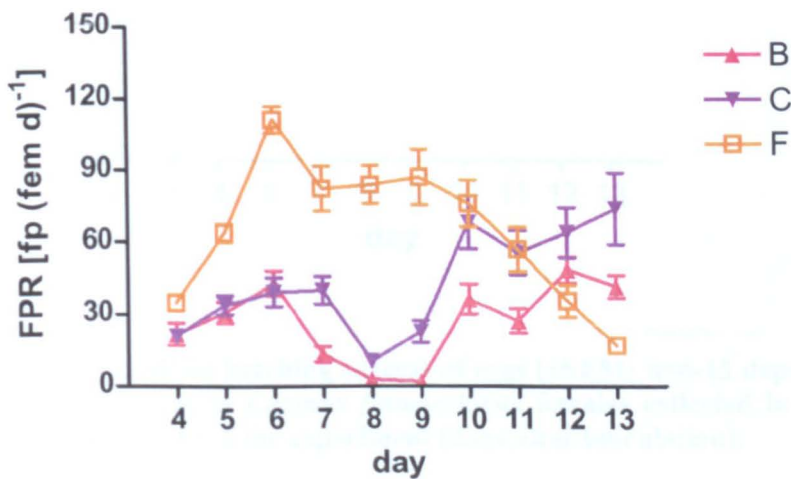


Figure 7-12: Mean faecal pellet production rates (FPR) ( $\pm$ SEM,  $n=12-15$  depending on day and treatment) [fp (fem d)<sup>-1</sup>] of *Calanus finmarchicus* females collected from mesocosm bags B, C, and F during the course of the experiment (days after inoculation).



Faecal pellet production rate (FPR) was significantly higher in mesocosm F compared to B (repeated measures ANOVA:  $F=6.23$ ,  $r^2=0.41$ ,  $p=0.0088$  and Tukey's posttest:  $p<0.01$ ) (Figure 7-12).

Hatching success and the percentage of abnormal nauplii, on the other hand, did not differ significantly between treatments (repeated measures ANOVA:  $p>0.05$ ). Although diatoms were producing oxygenated fatty acid derivatives, hatching success remained high (Figure 7-13), whereas the production of abnormal nauplii was continuously low (Figure 7-14).

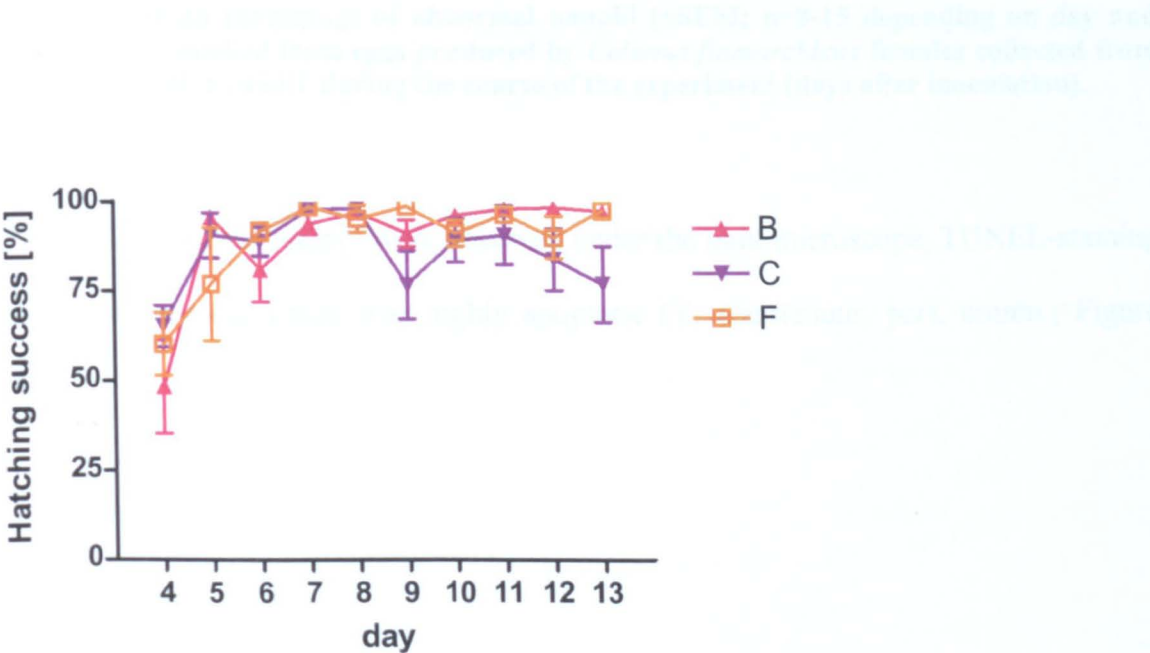


Figure 7-13: Mean hatching success of eggs ( $\pm$ SEM;  $n=6-15$  depending on day and treatment) [%] produced from *Calanus finmarchicus* females collected in mesocosm bags B, C, and F during the course of the experiment (days after inoculation).

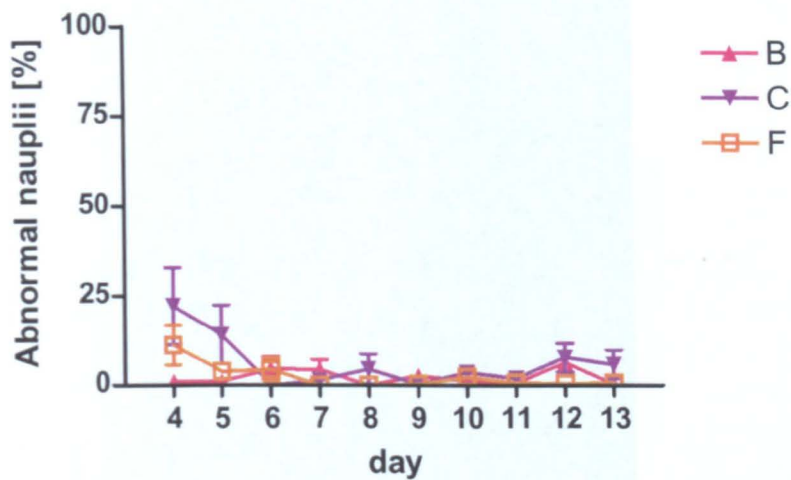


Figure 7-14: Mean percentage of abnormal nauplii ( $\pm$ SEM; n=8-15 depending on day and treatment) [%] hatched from eggs produced by *Calanus finmarchicus* females collected from mesocosm bags B, C, and F during the course of the experiment (days after inoculation).

Although no abnormal nauplii were observed under the light microscope, TUNEL-staining showed that naupliar tissue was highly apoptotic (Y. Carotenuto, pers. comm.; Figure 7-15).





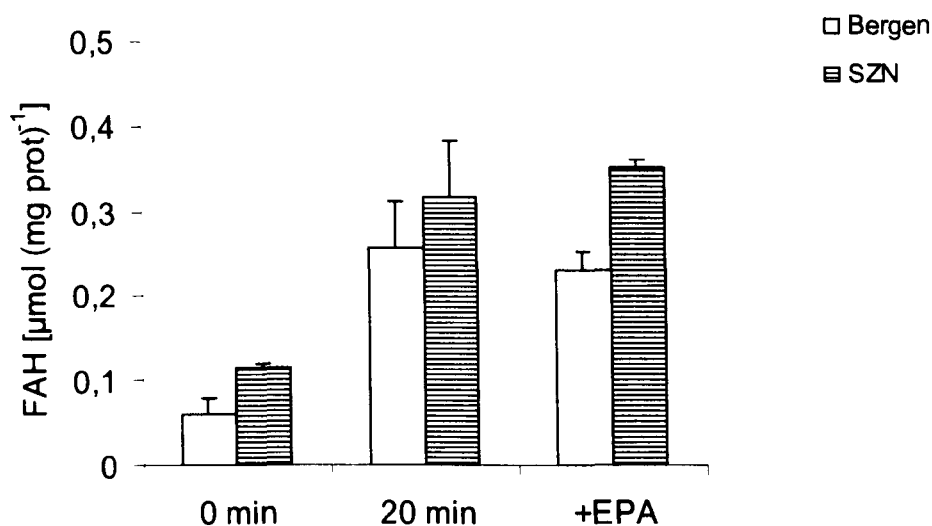
**Figure 7-15:** Nauplius of *Calanus finmarchicus* from the mesocosm experiment stained positive for apoptosis with TUNEL.

### 7.3.4 Culture Samples

The growth rate of the *Skeletonema marinoi* strain brought back from Bergen was  $0.37 \pm 0.11 \text{ d}^{-1}$ .

#### 7.3.4.1 Colorimetric Assay

In the colorimetric assay there was an increase of fatty acid hydroperoxides (FAHs) after 20 min compared to the blank in all cultures (Figure 7-16).

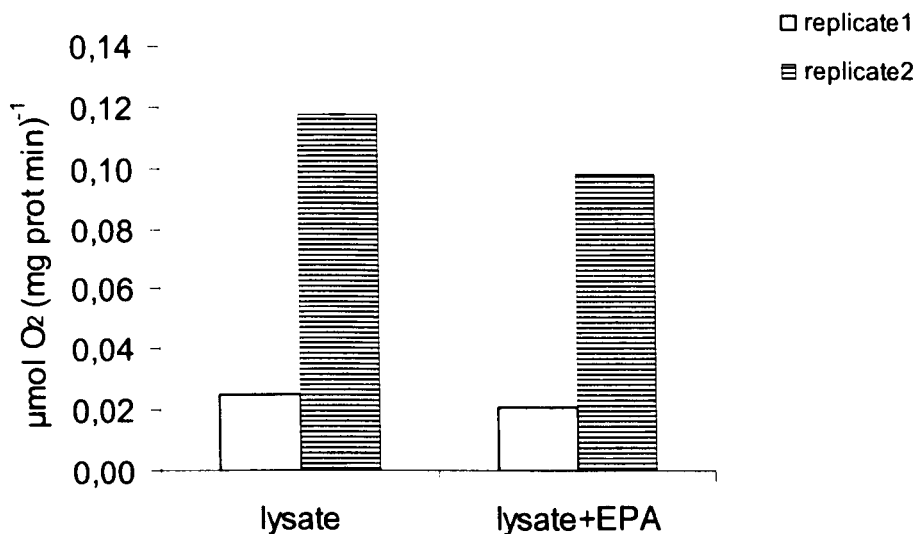


**Figure 7-16:** Mean concentrations (+SEM, n=2) of fatty acid hydroperoxide (FAH) normalized for protein [ $\mu\text{mol (mg prot)}^{-1}$ ] determined with the colorimetric assay in blanks (0 min), in cell lysate of the *Skeletonema marinoi* strain used for inoculation of the mesocosms, cultured in Bergen and at SZN (20 min), and in cell lysate upon EPA-addition (+EPA).

However, there seemed to be no difference in lipoxygenase (LOX) activity measured with the colorimetric assay between the cultures grown in Bergen and at SZN.

#### 7.3.4.2 Polarographic Assay

Only the two samples cultured at SZN were analyzed for lipoxygenase (LOX) activity also in the oxygraph. The first replicate showed lower oxygen consumption than the second (Figure 7-17).

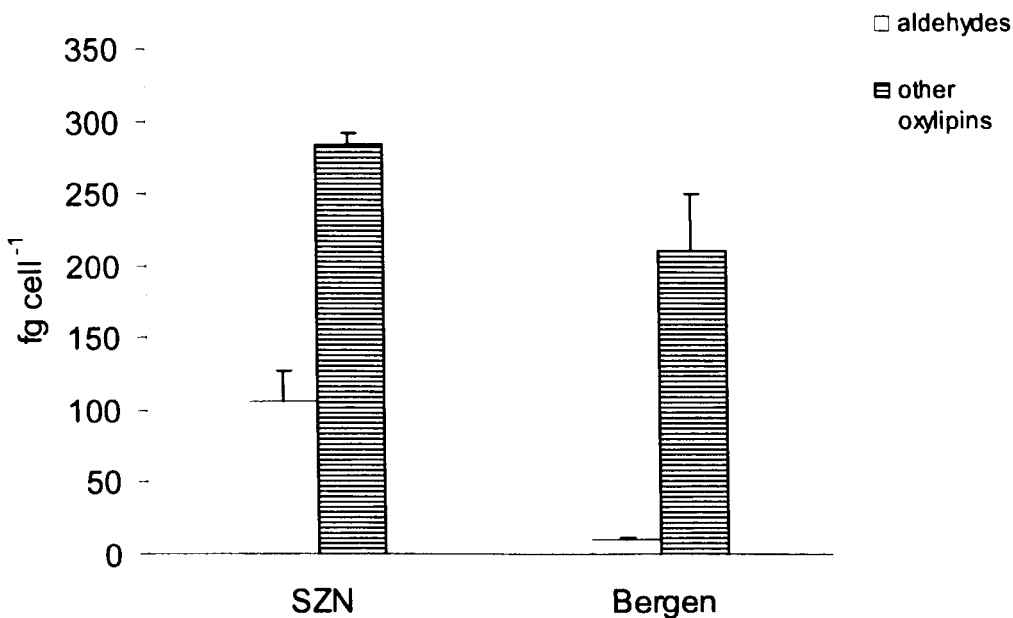


**Figure 7-17: Mean oxygen consumption rate (n=1) [ $\mu\text{mol O}_2 (\text{mg prot min})^{-1}$ ] of cell lysate (lysate) and cell lysate upon EPA-addition (lysate+EPA) in both replicates of the mesocosm strain of *Skeletonema marinoi* cultured at SZN determined at pH=8.15 in the polarographic assay.**

However, the response seemed to be the same in both replicates with no increase in oxygen consumption observable after the addition of exogenous EPA.

#### 7.3.4.3 Oxylipin Production

Oxylipin production seemed to be higher in the samples cultured at SZN (Figure 7-18).



**Figure 7-18: Production of aldehydes and oxylipins other than aldehydes (+SEM, n=2) normalized for cells [fg cell<sup>-1</sup>] in the mesocosm strain of *Skeletonema marinoi* cultured at SZN and in Bergen.**

This appeared to be mainly due to an increased aldehyde production, which was ten times higher in the cultures grown at SZN (106±28 fg per cell<sup>-1</sup>) than in the same strain cultured in Bergen (10±2 fg per cell<sup>-1</sup>). Production of oxylipins other than aldehydes did not seem to differ greatly between the two culture conditions.

As the negative effects on copepod reproduction during the mesocosm experiment were weak, the low aldehyde production in the Bergen cultures was examined more closely. Examining the different culture conditions at the two stations, it was discovered that different culture medium had been used. At SZN, diatoms were cultured on f/2 medium (Guillard 1975) prepared from natural filtered seawater. However, in Bergen Conway culture medium was used (Tompkins *et al.* 1995) which was prepared with slightly different nutrient concentrations (Table 7-7).

**Table 7-7: Comparison of Conway culture medium used in Bergen and f/2 medium used at SZN in respect to macro- (NO<sub>3</sub>, PO<sub>4</sub>) and micro- (Fe, Mn, Zn, Co, Mo, Cu) nutrient final concentrations [μM].**

C <sub>final</sub> [μM]	Conway (Bergen)	f/2 (SZN)
NO <sub>3</sub>	1176	882
PO <sub>4</sub>	167	36
Fe	5	18
Mn	1.8	0.9
Zn	0.015	0.077
Co	0.008	0.042
Mo	0.001	0.026
Cu	0.013	0.040

The Conway medium was richer in nitrate and phosphate compared to f/2, whereas f/2 contained higher concentrations of micronutrients, including iron, with the exception of manganese.

### 7.4 Discussion

In the two mesocosm bags inoculated with higher initial cell concentrations (Bags E and F), a bloom of *Skeletonema marinoi* developed. However, the initial inoculation was not quite successful. Cell counts showed that the initial concentration of *S. marinoi* in mesocosm F was ~500 instead of 1000 cells ml<sup>-1</sup>. Also in mesocosm E, initial cell concentrations differed slightly from the calculated cell concentrations. Actual measured cell concentrations were ~300 instead of 400 cells ml<sup>-1</sup>. Therefore, starting conditions in

mesocosms E and F were actually quite similar. Although the bloom in mesocosm F developed earlier and peaked at higher cell concentrations, bloom development in both mesocosms was indeed comparable.

*S. marinoi* produced fatty acid derived oxygenated metabolites in all mesocosms analyzed, even in the mesocosm bag in which *S. marinoi* cell concentrations remained low (Bag C). Aldehydes were not detected. A possible explanation for the lack of detectable aldehydes can obviously be lack of aldehyde production by the alga. This hypothesis was supported by low aldehyde production measured in culture samples of the strain used for inoculation. However, the same strain cultured at SZN showed high aldehyde production. The potential for aldehyde production was evidently present in this strain and possibly depended on nutrient conditions or other culturing parameters. Considering that particulate and dissolved aldehydes were indeed detected by a different method in this experiment, the extraction method applied in my case was probably not sensitive enough. As other oxylipins apart from aldehydes could be detected without problems, it is also possible that the long filtering necessary for obtaining the samples resulted in cell damage and loss of aldehydes which are less stable and more volatile than other oxylipins.

Cellular oxylipin production increased sharply during the demise of the bloom. Compared to values for oxylipin production obtained in culture with this strain of *S. marinoi* ( $0.2 \pm 0.06$  pg cell<sup>-1</sup>), values for cellular production in mesocosms at the end of the experiment were very high (6.5-11.5 pg cell<sup>-1</sup>). As suggested in Chapter 6, it is possible that lipoxygenase (LOX) enzymes continued to be active in seawater, although cells had lysed, leading to an overestimation of cellular production. Alternatively, higher cellular production in field compared to culture samples may have been due to an increased availability of substrate in field samples. Since all cells in the sample were lysed upon

sonication, fatty acid substrate from other sources apart from *S. marinoi* may have been available to LOX enzymes (Wichard *et al.* 2007). Another possible alternative source for oxylipins was other phytoplankton. In the mesocosms, *Phaeocystis sp.* in general was present in low amounts, but increased greatly towards the end of the bloom in mesocosm C. Samples from mesocosm C indeed showed high oxylipin production on day 12, even though *S. marinoi* was practically absent. *Phaeocystis pouchetii* (Hariot) Lagerheim has been found to produce aldehydes (Hansen *et al.* 2004) and therefore *Phaeocystis sp.* may have been contributing to the production of oxylipins in the mesocosms.

The modular production of oxylipins in *Pseudo-nitzschia delicatissima* during the different growth phases in culture with increases during the stationary and declining phase has been suggested as a regulatory mechanism (see Chapter 6). A similar pattern has been observed for the release of PUAs into the culture medium, which takes place in *S. marinoi* exclusively in the late stationary phase prior to culture decline (Vidoudez and Pohnert 2008). The increase in oxylipin production during the decline of the bloom in the present mesocosm experiment demonstrates for the first time, that a similar modulation of oxylipin production can be found in the field. Also in the mesocosm experiment, oxylipin production normalized for cells and chlorophyll *a* increased during the decline of the bloom. The similar pattern observed when normalizing oxylipin production for cells or chlorophyll can probably be explained by the fact that at least in mesocosms E and F chlorophyll was due mainly to *Skeletonema*, so that normalizing for chlorophyll gave again an indication of cellular production of oxylipins. This increase towards the end of the bloom suggests that oxylipins are involved in bloom termination, as has been suggested by Vardi *et al.* (2006).

The triggers for bloom termination are still under discussion and these results support the hypothesis that cells are regulating bloom termination themselves through cell-cell communication mediated by secondary metabolites (Vardi *et al.* 2006). Other possible explanations for the crash of the bloom in the present study include nutrient depletion, although only nitrates and phosphates were depleted at the end of the experiment, not silica, which may be more important for terminating diatom growth (Saito *et al.* 2006). Silica concentrations were lowest during the peak of the bloom and increased again towards the end, probably due to lysis of diatom cells. Cells at the end of the bloom were therefore most likely limited in nitrogen and phosphorus, but not silica. Sedimentation in the mesocosms cannot have been determining for bloom termination (Kiorboe *et al.* 1996), because in the 5 m deep water column, mixing was guaranteed by bubbling and no significant sedimentation was observed on taking down the mesocosms. Degradation by bacteria or viruses is a possible explanation for decreasing phytoplankton numbers in the present experiment because both bacterial and viral numbers increased in the mesocosms towards the end of the experiment (Bergkvist, in preparation). However, it is not clear if bacteria and viruses were the cause for cell lysis or merely profited from an increased pool of dissolved organic carbon due to cell lysis. Another possible explanation for a reduction of phytoplankton numbers could have been a top-down control due to grazing, even though the decline in cell numbers was quite sharp, making it questionable whether micro- or mesozooplankton grazing could have been that efficient in diminishing algal biomass.

Modulation of oxylipin production was found in all mesocosms, independent of *Skeletonema* concentration. LOX metabolism was apparently not affected by cell concentrations, but seemed to depend on the progression of the experiment. An increase in oxylipin production over time was found also in *Skeletonema* cells from the mesocosm inoculated only with nutrients (mesocosm C), in which no bloom of *Skeletonema*



developed. Ribalet *et al.* have demonstrated that *S. marinoi* increases aldehyde production under phosphate and nitrate limitation (Ribalet *et al.* 2007b). In all mesocosms, nitrates and phosphates were depleted at the end of the experiment. Therefore, the increase in oxylipin production may have been due to nutrient stress encountered by cells at the end of the bloom, possibly inducing a decline in cell numbers under unfavourable conditions.

The effect of oxylipin production by *S. marinoi* on copepod embryonic development was weak in the mesocosm experiment. Even though *S. marinoi* abundance was 100-fold in mesocosm F compared to C or B, this had neither an impact on hatching success of copepod eggs nor on naupliar development. The only parameter demonstrating a toxic effect of *S. marinoi* on copepod reproduction was the occurrence of apoptosis in naupliar tissue. Apoptotic tissue was an indication of internal lesions due to toxic metabolites (Romano *et al.* 2003), even though the effect was not strong enough to induce abnormalities in nauplii. One explanation for the weak effect on copepod reproduction may be that copepods were feeding on alternative foods. However, copepods tend to produce large amounts of faecal pellets when feeding on *S. marinoi* (personal observations) and therefore the high faecal pellet production observed in females fed phytoplankton from mesocosm F suggests that copepods were indeed feeding on *S. marinoi*. This also seems likely considering that *S. marinoi* was the main phytoplankton species available for grazing. Another explanation for continuing high reproductive success may be the short duration of the bloom. This short duration may have been enough to induce apoptosis, but was too short to decrease hatching success of eggs. Also in the laboratory, it can take days up to weeks to establish a deleterious effect of oxylipin-producing diatoms on copepods (d'Ippolito *et al.* 2002b; Ceballos and Ianora 2003). Furthermore, it is possible, that the population of *Calanus finmarchicus* females used in the incubation experiments was rather insensitive to oxylipins, because the response of different copepod species to the same

diatom species can vary (Paffenhoefer *et al.* 2005) and high reproductive success of *C. finmarchicus* during diatom blooms has been observed previously in the field (Ban *et al.* 1997; Koski 2007).

Laboratory studies on the *S. marinoi* strain used for the present mesocosm experiment gave interesting indications as to the variability in oxylipin metabolism between strains and those due to culture conditions. Growth rate in this strain ( $0.37 \pm 0.11 \text{ d}^{-1}$ ) was about half of the growth rate determined for the Adriatic *S. marinoi* ( $0.76 \pm 0.04 \text{ d}^{-1}$ ), which was presumably due to the lower cultivation temperature (10°C instead of 20°C). Interestingly, oxygen consumption rate in this strain was not increased by the addition of eicosapentaenoic acid (EPA) to the cell lysate, even though oxygen consumption of the cell lysate was high. This high oxygen consumption rate of the cell lysate was presumably due to LOX activity on endogenous fatty acids, but LOX enzymes were apparently not using exogenous EPA. Therefore, in the present samples the polarographic assay could not demonstrate LOX activity, at least not how originally defined as the increase in oxygen consumption rate upon EPA-addition to the cell lysate (see Chapter 3). Apart from these apparent peculiarities of the Norwegian *S. marinoi* strain, there also seemed to be an influence of culture conditions on oxylipin production. Although there were no significant differences in fatty acid hydroperoxide production detected with the colorimetric assay, oxylipin production was apparently greater in the culture grown at SZN compared to the one grown in Bergen. This may have been due to the different culture media used with lower concentrations of the macronutrients nitrate and phosphate used at SZN.

## 7.5 Conclusions

In the present mesocosm experiment, a mono-specific bloom of *Skeletonema marinoi* was successfully induced in the two mesocosm bags inoculated with higher initial concentrations of *S. marinoi*. *S. marinoi* was producing aldehydes as well as oxylipins other than aldehydes in all mesocosms examined with a peak at the end of the bloom when values were normalized for cells. This indicates an involvement of oxylipins in bloom demise and gives some of the first field evidence for the role of oxylipins as growth regulators. Oxylipin production normalized for carbon was highest during the peak of the bloom. However, there was only a weak effect of oxylipin production on copepod reproductive success. Although there was some indication for a detrimental impact in the form of apoptotic nauplii, hatching success of copepod eggs remained high. A possible explanation may be the short duration of the bloom, which succeeded in inducing apoptosis in hatched nauplii, but was not strong enough to significantly impact copepod reproduction.

## 8 Conclusions

In this thesis, lipoxygenase (LOX) activity and oxylipin production in several diatom species, with special emphasis on *Skeletonema marinoi*, were examined in different systems. Oxylipin metabolism in diatoms was studied in laboratory cultures, under semi-controlled conditions in a mesocosm and in the field. Furthermore, the influence of different factors, such as nutrient-stress and different growth phases, has been examined. An interesting aspect that has emerged from all of these studies is the lack of correlation between LOX activity assays and oxylipin production.

### 8.1 Methodological Aspects

The original aim of the thesis to develop an easy-to-use, simple assay for oxylipin production in phytoplankton samples based on lipoxygenase (LOX) activity has not proved feasible. In part this was due to a pH-dependency of the involved enzymatic reactions. Especially in *S. marinoi*, LOX activity was often low or absent in the cell lysate at the pH of the colorimetric assay (pH=6.0), which precluded using this assay for accurately determining LOX activity. However, also LOX activity measured at pH=8.15 in the polarographic assay showed no continuous correlation with oxylipin production. These findings underline the importance of unifying protocols, making it best to carry out extractions for oxylipin quantification at pH=8.15 to be able to compare oxylipin production more easily with LOX activity as determined with the polarographic assay. One may also carry out extractions at pH=6.0 to compare oxylipin production to LOX activity measured with the colorimetric assay, but presumably LOX enzymes will be active at pH=8.15 in nature and therefore extracting at this pH should give a more ecologically relevant value. Additionally, in *S. marinoi* suspending cells at pH=6.0 led to lower

production of oxylipins compared to suspending cells at pH=8.15, thereby demonstrating that carrying out extractions at pH=6.0 may underestimate oxylipin production in certain diatom species.

Although the oxygraph was more applicable to detailed physiological studies in diatoms, being a direct measurement of LOX activity, it was limited by its lack of specificity. The polarographic assay could only demonstrate LOX activity on the exogenous fatty acid used in the assay, in this case eicosapentaenoic acid (EPA). In the diatoms *Pseudo-nitzschia delicatissima* and *Chaetoceros affinis*, adding only EPA to the cell lysate did not underestimate LOX activity because these species seem to use only C<sub>20</sub>-fatty acids as LOX substrate (Fontana *et al.* 2007b; d'Ippolito *et al.* 2009). However, since *Thalassiosira rotula* and *Skeletonema marinoi* metabolize C<sub>16</sub>- as well as C<sub>20</sub>-fatty acids (d'Ippolito *et al.* 2003; d'Ippolito *et al.* 2005), it would be wise to carry out the polarographic assay with both of these substrates to evidence the entire LOX metabolism in these diatom species. To this end, it is important to determine the composition of oxylipins in the diatom species to evaluate the relative contribution of each pathway to total oxylipin production. The polarographic assay with cell lysates of *S. marinoi* would be best carried out with a 2:1 mixture of C<sub>20</sub>- to C<sub>16</sub>-fatty acids, as this would correspond to the relative relationship of oxylipins deriving from these precursors in this species.

As the polarographic assay was more time consuming with 15 min needed for one replicate, the colorimetric assay was preferred for a rapid screening of LOX activity in samples. However, certain improvements should be taken into consideration in the future, such as establishing the range of linearity before reading samples and developing an alternative for reading blank values of the cell lysate. Furthermore, it should be examined more closely, whether the colorimetric assay can be applied to dosing fatty acid

hydroperoxide (FAH) concentrations in phytoplankton samples, even in the absence of LOX activity at pH=6.0, by respecting only the 5 min necessary for colour development (Anthon and Barrett 2001).

## 8.2 Uncorrelated Lipxygenase Activity and Oxylin Production

The lack of correlation between lipxygenase (LOX) activity and oxylin production gave interesting insights into oxylin metabolism in diatoms. Although the oxidation of fatty acids by LOX enzymes is surely an important step in the formation of oxylins, it is apparently not the only determining reaction. Enzymes responsible for the conversion of fatty acid hydroperoxides (FAHs), e. g. lyases, peroxidases, and allene oxide synthases (Andreou *et al.* 2009) and their differential expression may be just as important in determining the quantitative and qualitative production of oxylins, as the initial oxidative reaction. Enzymatic systems for converting FAHs into other oxylins should therefore be considered more closely in further research, because these may be determining in the production of end-metabolites, such as aldehydes, hydroxy-acids and epoxyalcohols. Also non-enzymatic reactions need to be taken into consideration, which may be involved in the transformation processes, e. g. in the reduction of FAHs to hydroxy-acids (Andreou *et al.* 2009). Another possible explanation for a lack of correlation between FAH and oxylin production would be the presence of a bifunctional LOX, which directly converts fatty acids into secondary oxylins without releasing intermediate FAHs. Such an enzyme has been suggested for diatoms (Pohnert 2005) and may explain a lack of FAH detection in the presence of oxylin production.

The present results also strongly indicate that there exist still other secondary metabolites deriving from the oxidative metabolism of fatty acids in diatoms which need to be characterized in order to correlate the oxidation of fatty acids, hence LOX activity, to the production of oxylipins. The lack of correlation between the production of FAH intermediates, measured in the colorimetric assay, and the production of known end-metabolites, the oxylipins, cannot be due just to changing LOX activity between the pH of the colorimetric assay (pH=6.0) and the pH of extraction for oxylipin quantification (pH~7.0). As FAHs are instable and are therefore rapidly transformed into secondary oxylipins, the lack of correlation between FAH production and oxylipin production strongly indicates the presence of unknown metabolites. Apparently, the production of aldehydes and other oxylipins as end-metabolites of oxidative fatty acid metabolism does not yet allow for a complete picture of the mode of action of *Skeletonema marinoi*.

However, the production of FAHs, measured in the colorimetric assay, did seem to correlate directly with copepod hatching success during the diatom bloom in the Northern Adriatic Sea in 2005 (see Chapter 2). This direct effect may be mediated by a toxic effect of FAHs, but another explanation is also feasible. Depending on the specificity of the assay for FAHs, the colorimetric assay may be more appropriate for demonstrating a general oxidative burst triggered by cell damage. If the negative effect of fatty acid oxidative metabolism on copepod reproduction is due rather to a general increase in oxidative stress than to the action of specific metabolites (Fontana *et al.* 2007b) or if other metabolites are still involved in this effect, the colorimetric assay could give us a more general view of the potential impact of diatoms. It therefore seems useful to continue using the colorimetric assay for the analysis of field phytoplankton samples, especially if it can be adapted to dosing FAH concentrations in samples, as this assay may give a more general view of the potential impact of diatoms due to the oxidative metabolism of fatty acids.

Whether LOX activity correlates to oxylipin production may also depend on the complexity of oxylipin metabolism. In *Pseudo-nitzschia delicatissima*, only one LOX activity has been described so far, a 15(*S*)-LOX which oxidizes C<sub>20</sub>-fatty acids, leading to the production of hydroxy-acids, epoxyalcohols, and  $\omega$ -oxo-acids (see Chapter 6). In this diatom species, there was a good correlation between LOX activity determined in the polarographic and colorimetric assays and the production of end-metabolites. The applicability of the colorimetric assay was due to LOXs being active at pH=6.0 and the good correlation between LOX activity and oxylipin production observed in this species was possibly due to a simpler, more straight-forward oxylipin metabolism in this species compared to e. g. *S. marinoi*.

### 8.3 Diversity of Oxylipin Metabolism among Clones

Clones of *Skeletonema marinoi* were isolated during the diatom bloom in the Northern Adriatic Sea during different years and analyzed for oxylipin metabolism in the laboratory (see Chapter 4). The strong reduction in hatching success observed in the field in 2003 (see Chapter 2) may have been due to a strong production of fatty acid hydroperoxides (FAHs) observed in the *S. marinoi* clone isolated in that year. However, strong FAH production was not observed in the isolate from 1997, even though there had been a comparably strong effect on hatching success in that year (Miralto *et al.* 1999). Strong reduction of hatching success in 1997 seemed to correlate instead to strong oxylipin production in this clone. This clone seemed to produce the largest amount of aldehydes of all the clones analyzed. The stronger toxic effect of aldehydes compared to other oxylipins (Fontana *et al.* 2007b) may explain the strong reduction in hatching success observed at sea during the diatom bloom in that year (Miralto *et al.* 1999). The low aldehyde production in the clone



isolated in 2005 also seemed to correlate to the low aldehyde production observed in the phytoplankton samples collected in that year (see Chapter 2). However, oxylipin production did not differ greatly between the isolates from 2003 and 2004, whereas hatching success was impacted more strongly in 2003 than in 2004.

Apparently, no direct comparisons can be made between the oxylipin metabolism analyzed in the laboratory of a specific clone and the oxylipin metabolism of the bloom from which the clone has been isolated. Isolating single cells from the bloom does not guarantee isolating the most abundant clone responsible for the bloom of that year. Additionally, recent studies demonstrate large genetic diversity within blooms (Medlin *et al.* 1999; Rynearson and Armbrust 2000), making it unlikely that one clone will represent an entire bloom. Therefore, it is more realistic to analyze field phytoplankton samples from a given year, as was done in Chapter 2, rather than rely on culture studies of isolated clones. Another interesting possibility would be to carry out incubation experiments in the laboratory with different clones to validate whether differences in lipoxygenase (LOX) metabolism are the causative agents for variations in copepod reproductive success between different years, as was indicated by Pohnert *et al.* (2002) when two strains of *Thalassiosira rotula* with different potentials for aldehyde production were fed to copepods.

The ecological significance of oxylipins is still under discussion and may lie in defence from grazers (Miralto *et al.* 1999; Fontana *et al.* 2007b), in the ability to outcompete bacteria (Ribalet *et al.* 2008) or other phytoplankton (Casotti and Mazza 2005; Ribalet *et al.* 2007a), on the possibility to induce programmed cell death under unfavourable conditions (Vardi *et al.* 2006) or on a multitude of these factors. Independent of the mode of action of these metabolites, variations in LOX activity or oxylipin production may

therefore impart an evolutionary advantage to certain clones of the same species which may be preferentially selected for.

## 8.4 Functions of Oxylipins in Marine Ecosystems

The different functions suggested for oxylipins in the marine ecosystem may shape interactions between trophic levels (Miralto *et al.* 1999), within trophic levels (Ribalet *et al.* 2007a) and even within populations (Vardi *et al.* 2006). The possible regulatory role of oxylipins in bloom termination is an interesting new concept (Vardi *et al.* 2006), which merits further study as the reasons and triggers for the decline of phytoplankton blooms are still under discussion. The hypothesis that oxylipins may trigger bloom decline is based on observations obtained in the laboratory from culture studies as in Chapter 6 (Vardi *et al.* 2006; Vidoudez and Pohnert 2008). The results obtained from the mesocosm study in this thesis indicate that the same phenomenon can be found in nature, even though increased oxylipin production towards the end of blooms could not be verified in the field during the *Skeletonema* bloom in the Northern Adriatic Sea. On the contrary, in that study, increased production of oxylipins was observed at the beginning of the bloom period. Producing large amounts of oxylipins at the beginning or shortly before the bloom may be beneficial for diatoms. Overwintering copepods rising to the surface and beginning to feed in late winter may be impacted by these oxylipins produced by diatoms during this time period, leading to reduced hatching success and a decreased next-generation grazer population. This way grazing pressure may be reduced during the following bloom period. Field data on oxylipin production during diatom blooms are still scarce and it will be interesting to see, whether modulated oxylipin production is relevant in the field and whether increased production may be beneficial at the beginning of blooms, supporting the hypothesis of

oxylipins as grazer defence, or whether high oxylipin production at the end of blooms may be involved in bloom termination. A possible trigger for initiating increases in oxylipin production leading to bloom demise may be nutrient limitation, as indicated by laboratory studies (Ribalet *et al.* 2007b) and by the nutrient depletion in the mesocosm experiment (see Chapter 7). This way, a co-ordinated decline in cell numbers may be triggered by unfavourable conditions. An increased oxylipin production due to nutrient stress could not be verified for phosphorus-stress in this thesis. However, this may have been due to control cultures being close to phosphorus (P)-limitation or to P-limitation being a weak stress inducer.

The results obtained for *S. marinoi* grown under P-limitation in continuous culture validated the strong deleterious effect of this diatom species on calanoid copepods, which was also observed for the *S. marinoi*-dominated bloom in the Northern Adriatic Sea in 2005. These results support the hypothesis that oxylipins act as defence molecules, negatively impacting grazer reproductive success in the laboratory, as well as in the field. However, the results obtained from the mesocosm experiment also highlight, that the extent of negative effects induced by maternal diatom diets on copepod reproduction may depend on numerous factors, such as the duration of a diatom bloom. The results obtained from the Northern Adriatic Sea also suggest that individual cellular oxylipin production of diatoms is more important for copepods than the overall potential for oxylipin production of the phytoplankton assemblage. Another important point to consider is the variable sensitivity of different copepod species, which may be due to differential expression of antioxidant enzymes such as catalases and glutathione-S-transferases (Lee *et al.* 2007; Souza *et al.* 2007). Some indications for a molecular response to oxidative stress by increased expression of antioxidant genes have been found in *Calanus finmarchicus* (Hansen *et al.* 2008). Possible “detoxification” mechanisms of copepods against oxidative

stress need to be examined further, as these may substantially contribute to understanding variability of copepod response in the field.

## 8.5 Closing Remarks

In short, there is much we still need to learn about oxylipin metabolism in diatoms. Further studies should be carried out to better understand the discrepancy of fatty acid hydroperoxide (FAH) production and oxylipin production and the possible presence of unknown secondary metabolites should be taken into consideration to close this gap. Therefore, lipoxygenase (LOX) activity assays cannot substitute oxylipin analyses. Both assays, the colorimetric and the polarographic assay, are valid for determining LOX activity, if certain considerations are taken into account and if the assays are adapted to the various systems. Analysing LOX activity and oxylipins is complementary, highlighting different aspects of oxylipin metabolism. Whereas oxylipin analyses are very specific, the colorimetric assay may provide a broader view of the oxidative potential of diatoms, also because oxylipin analyses are limited by the identification and characterization of oxylipin compounds, thereby not taking into account unknown metabolites. Whereas diatoms undoubtedly negatively impact the reproductive success of calanoid copepods, many factors need to be taken into account to understand diatom-copepod interactions in the field and diversity of oxylipin metabolism is an important aspect for understanding these interactions. Evidently, we do not yet have the complete picture of fatty acid oxidative metabolism in diatoms, which is turning out to be much more complex than originally envisioned.

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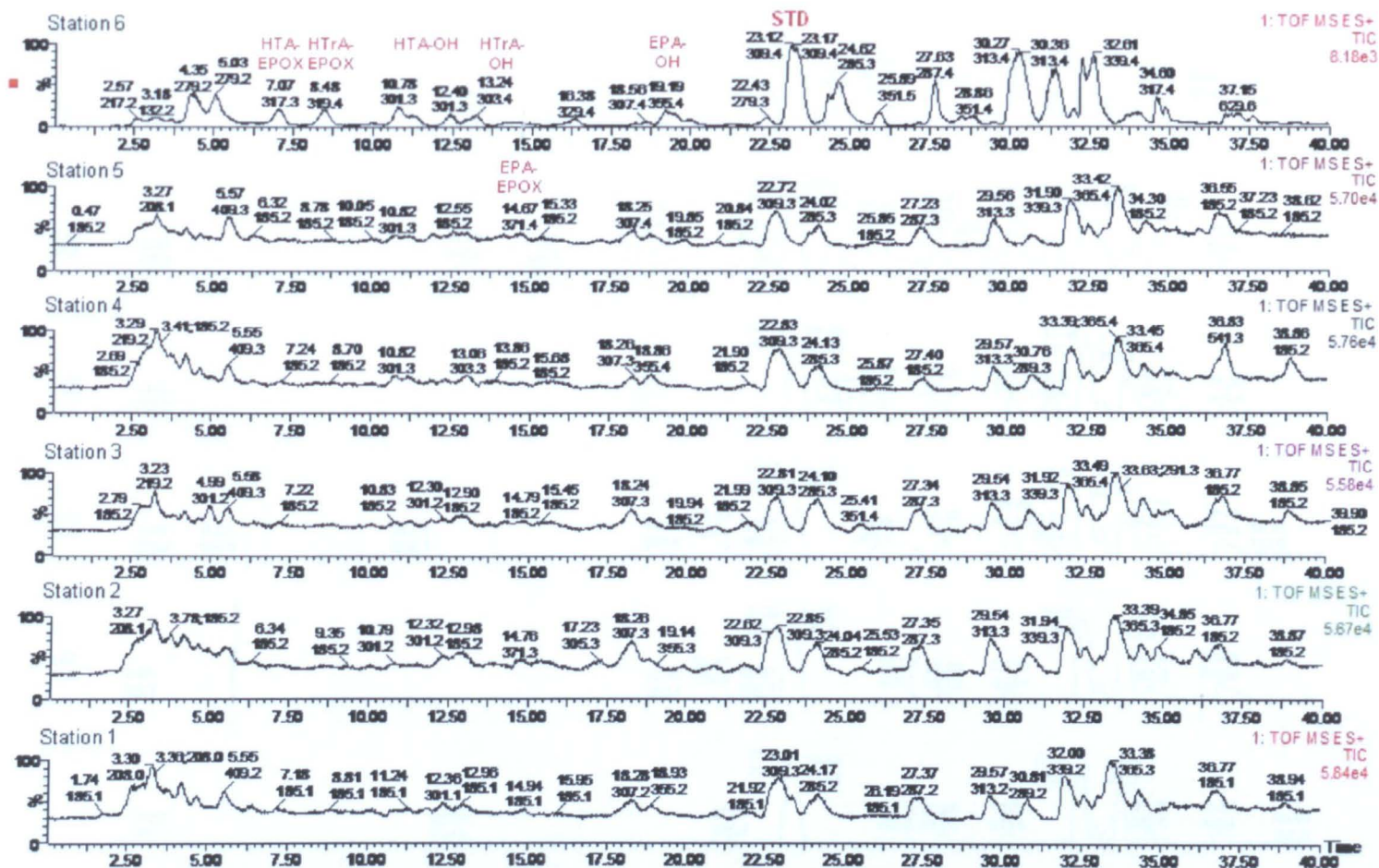
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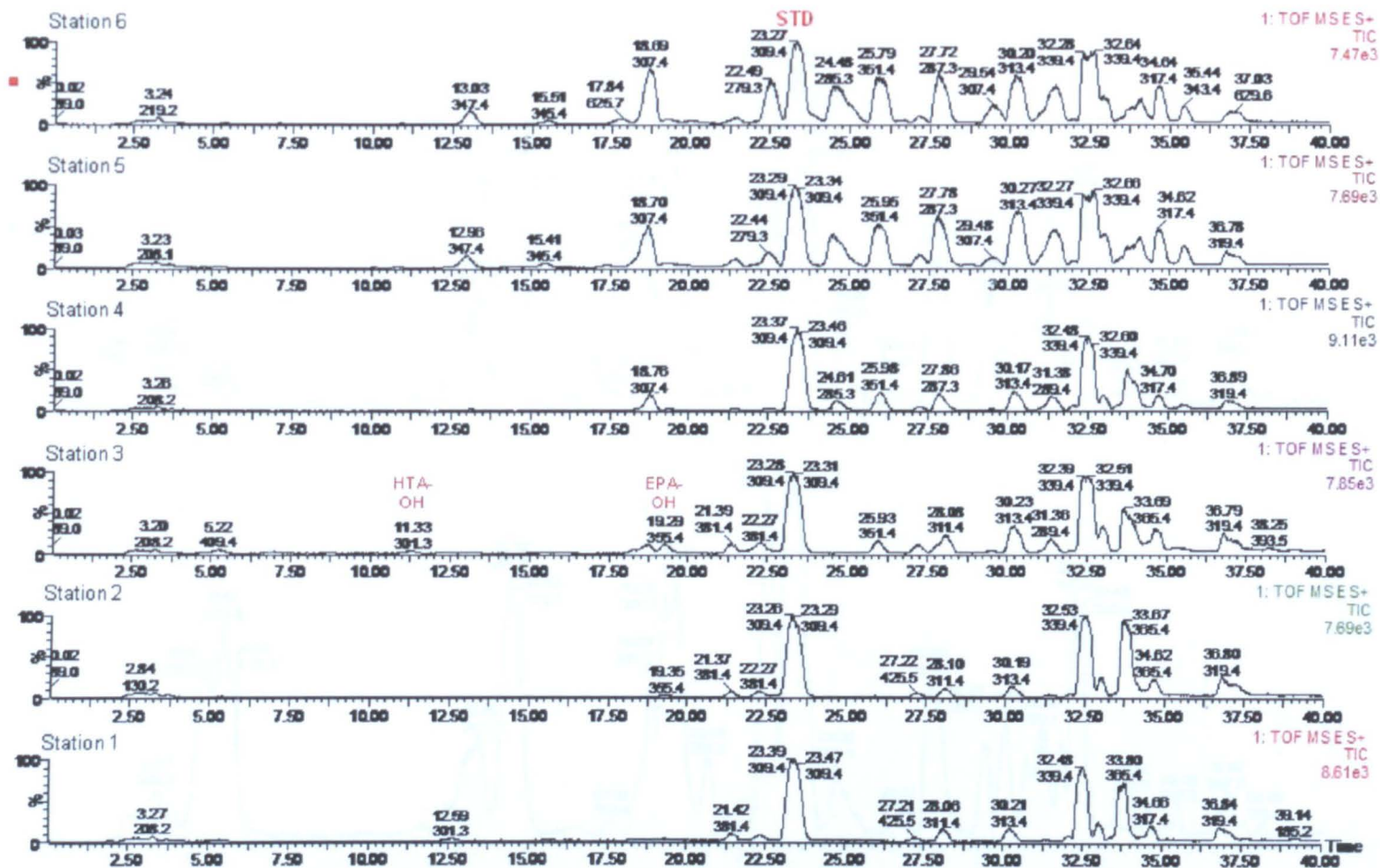
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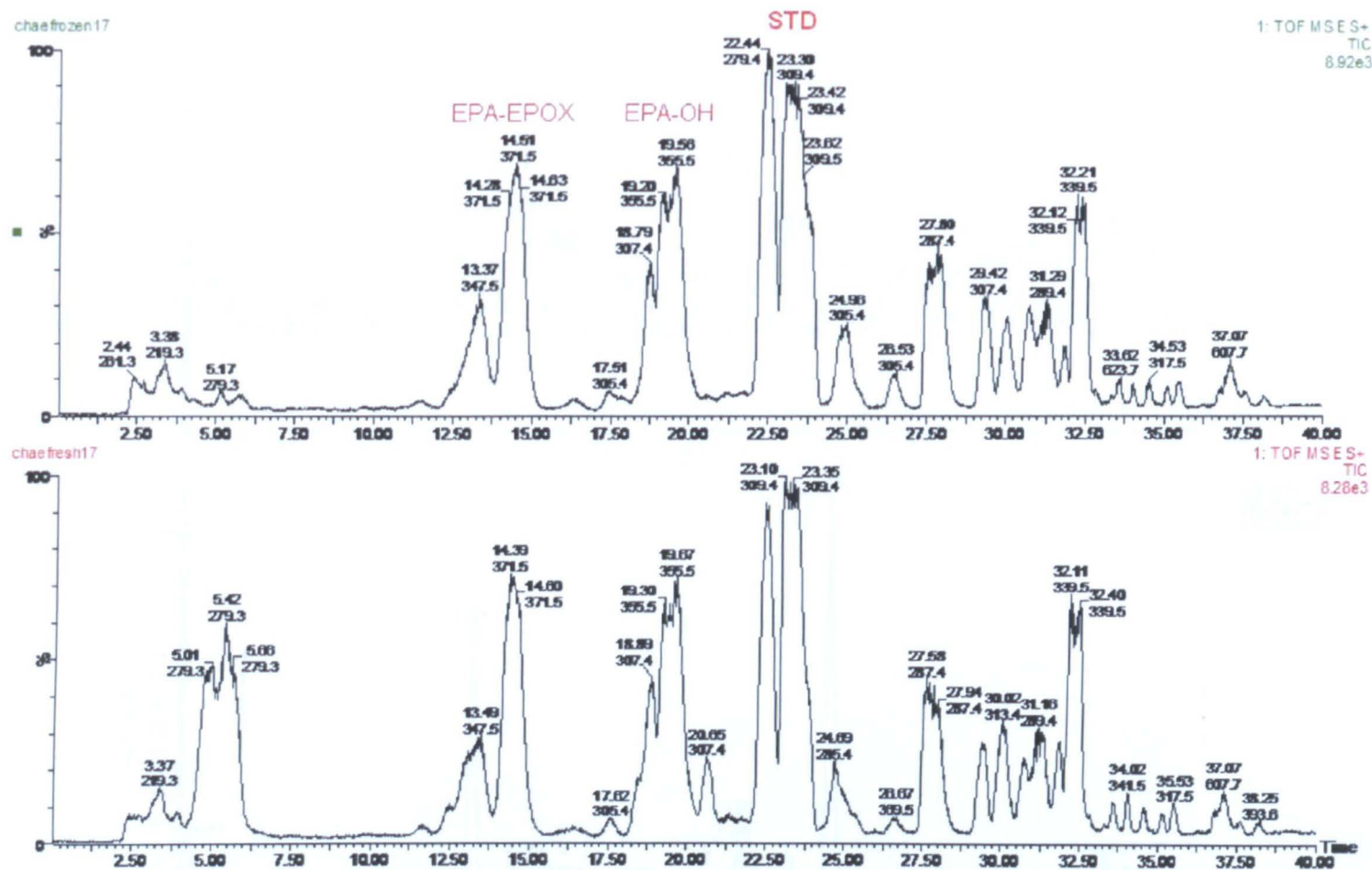
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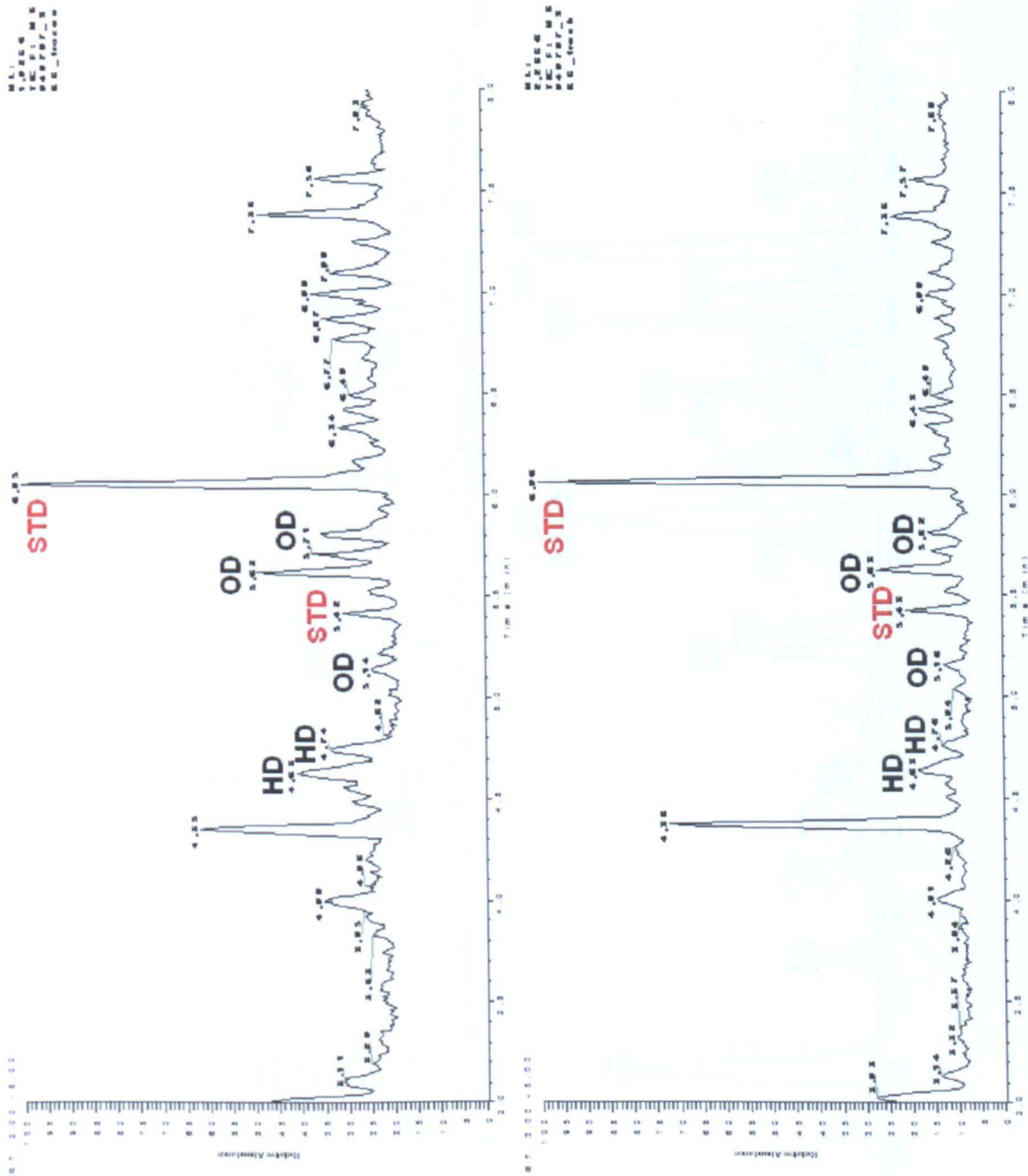
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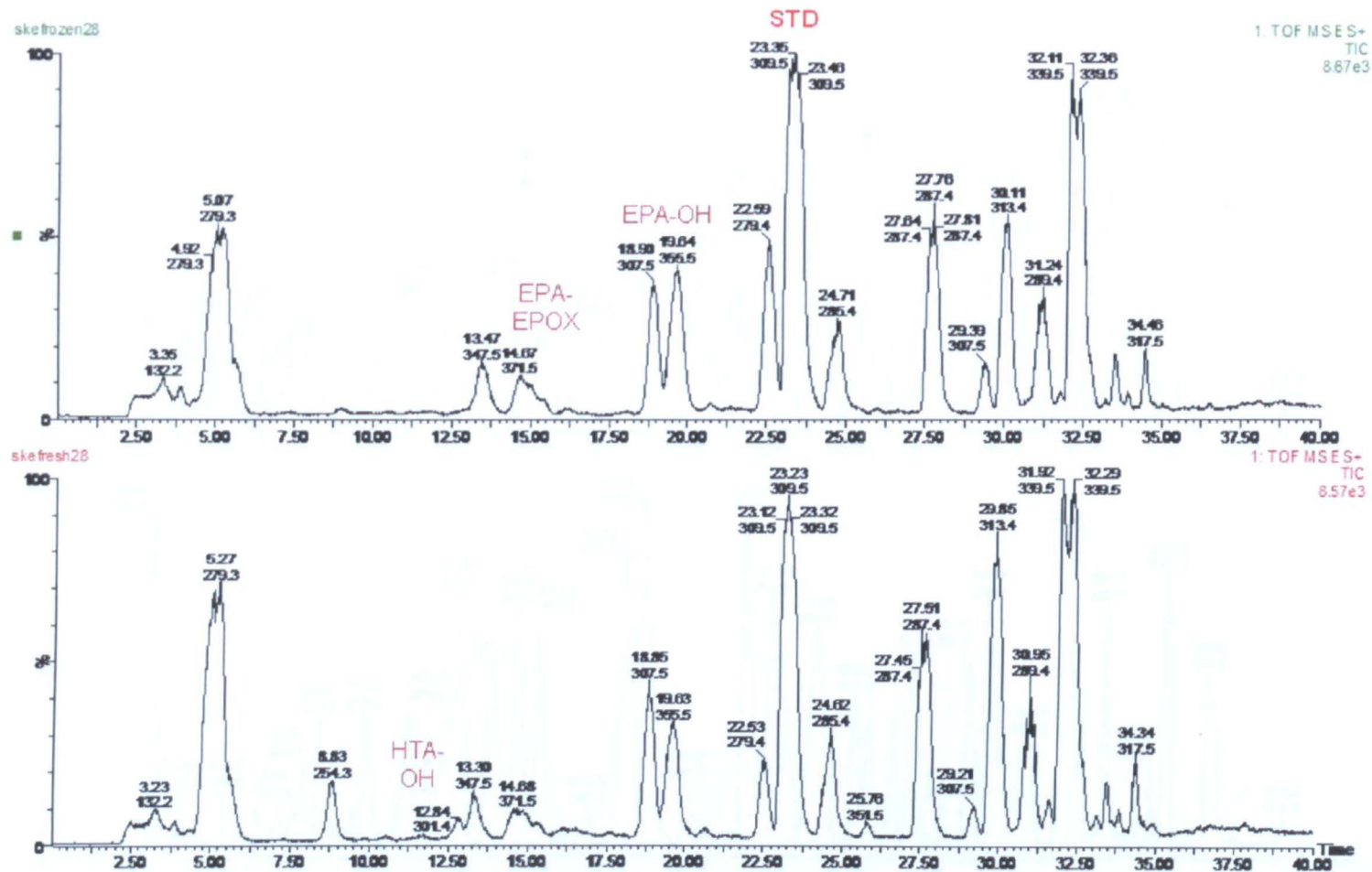


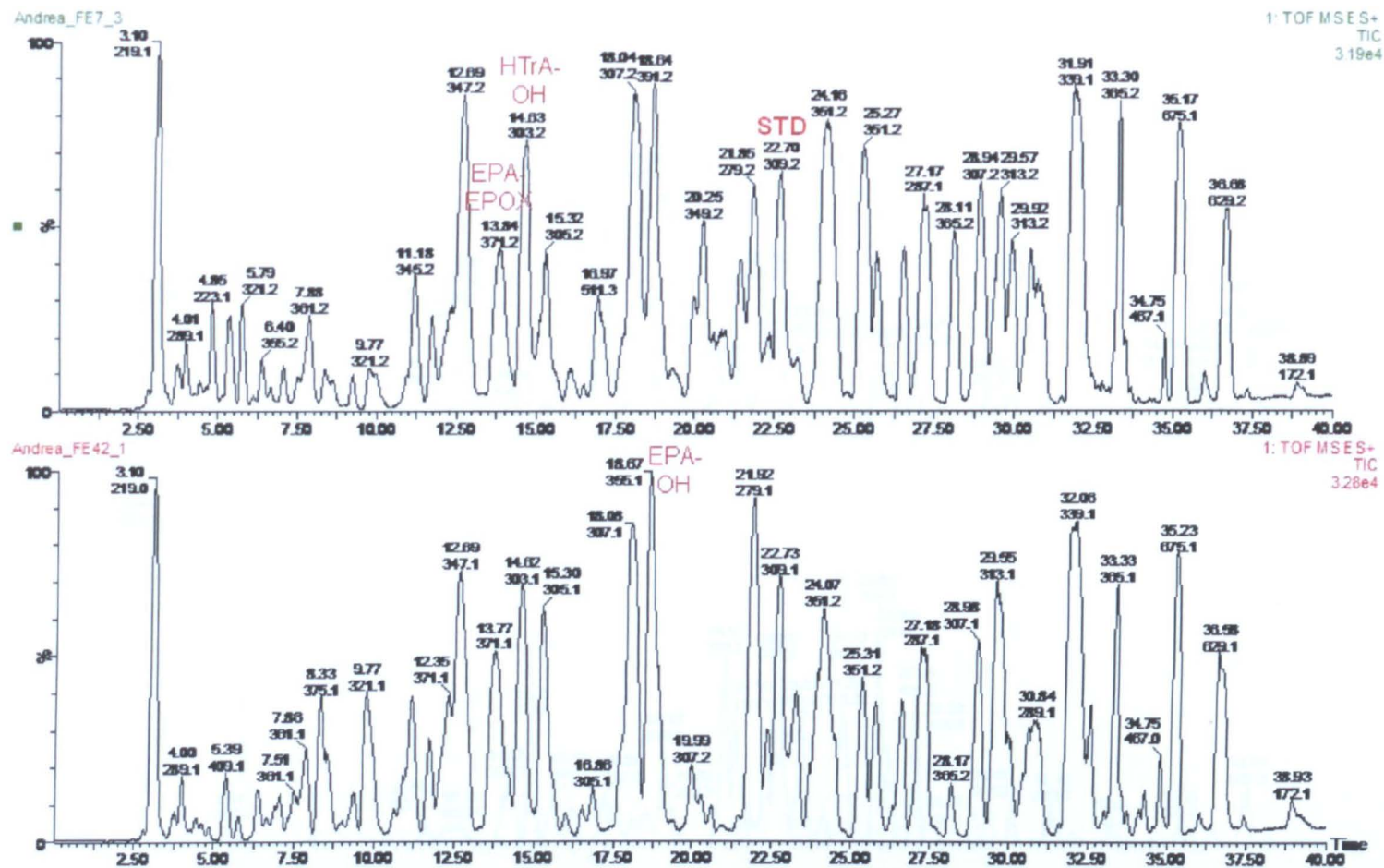


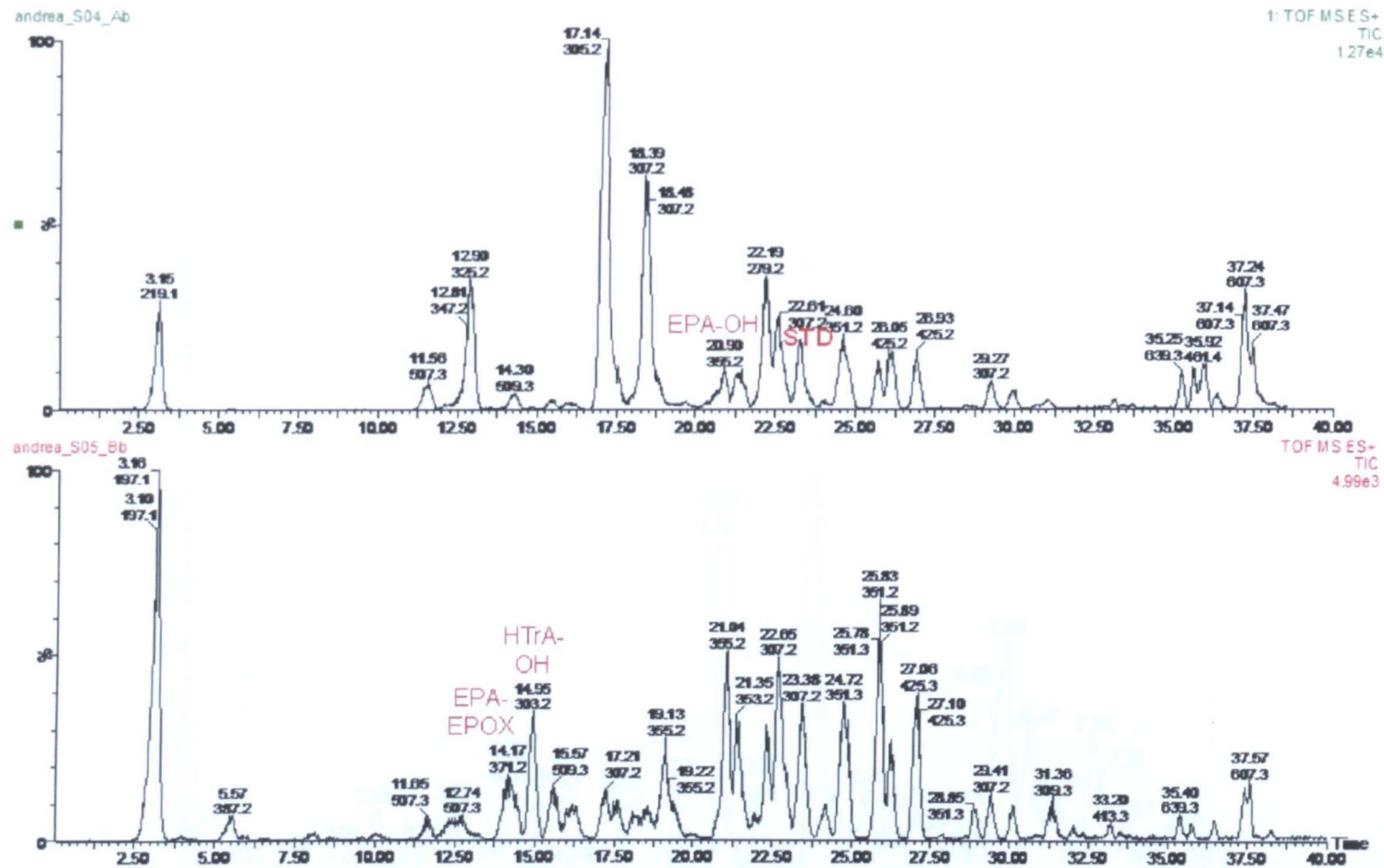






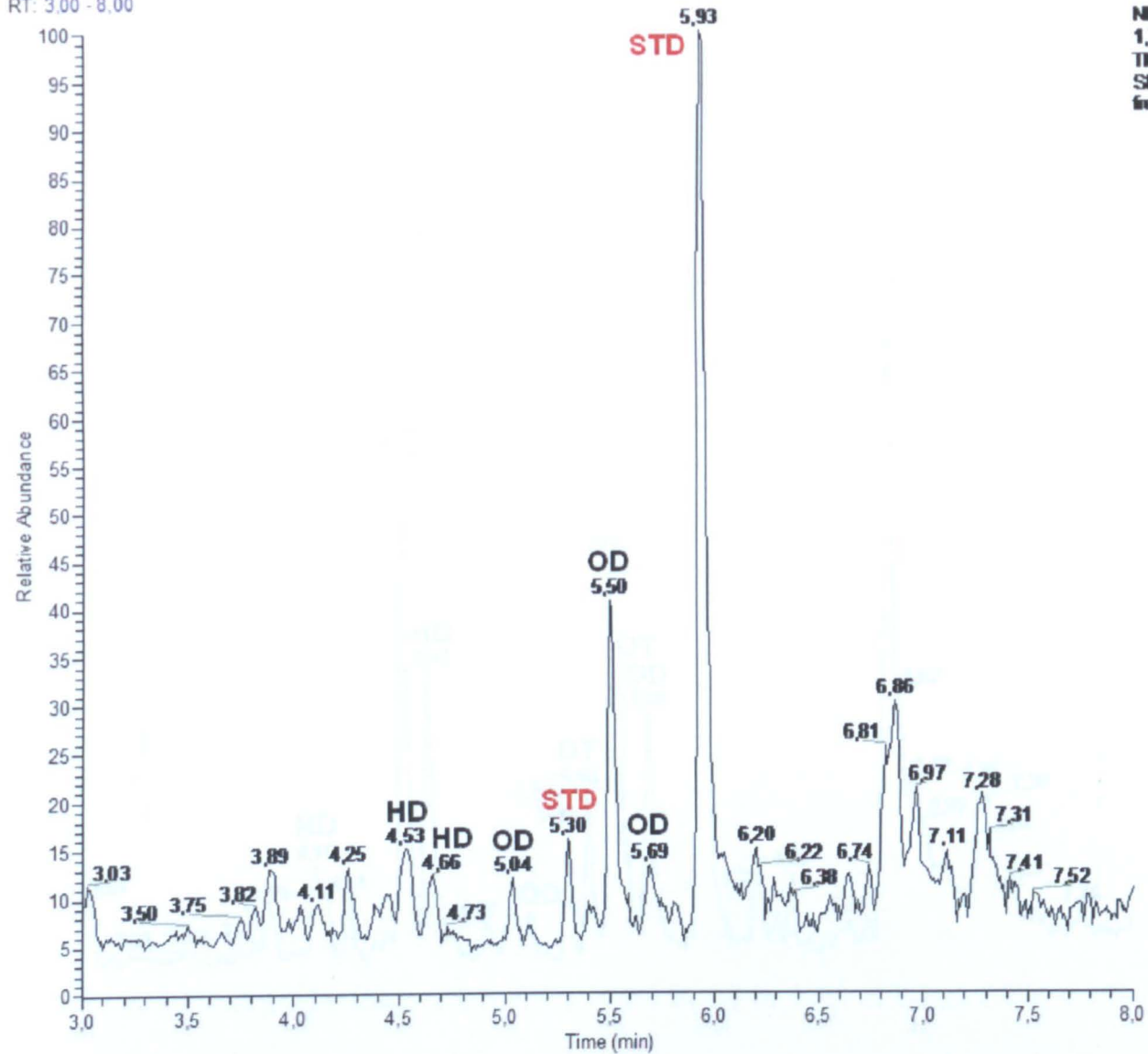








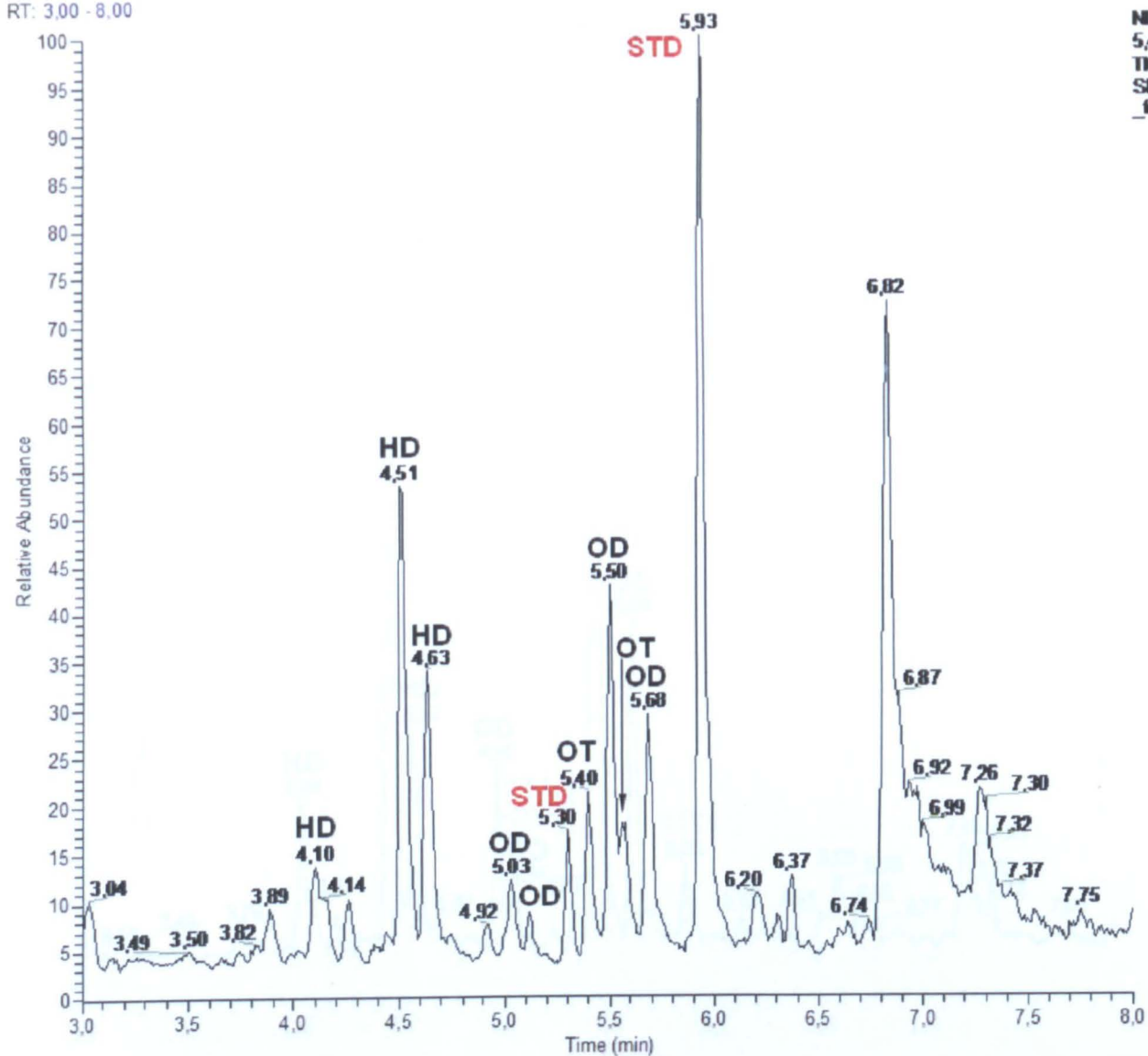
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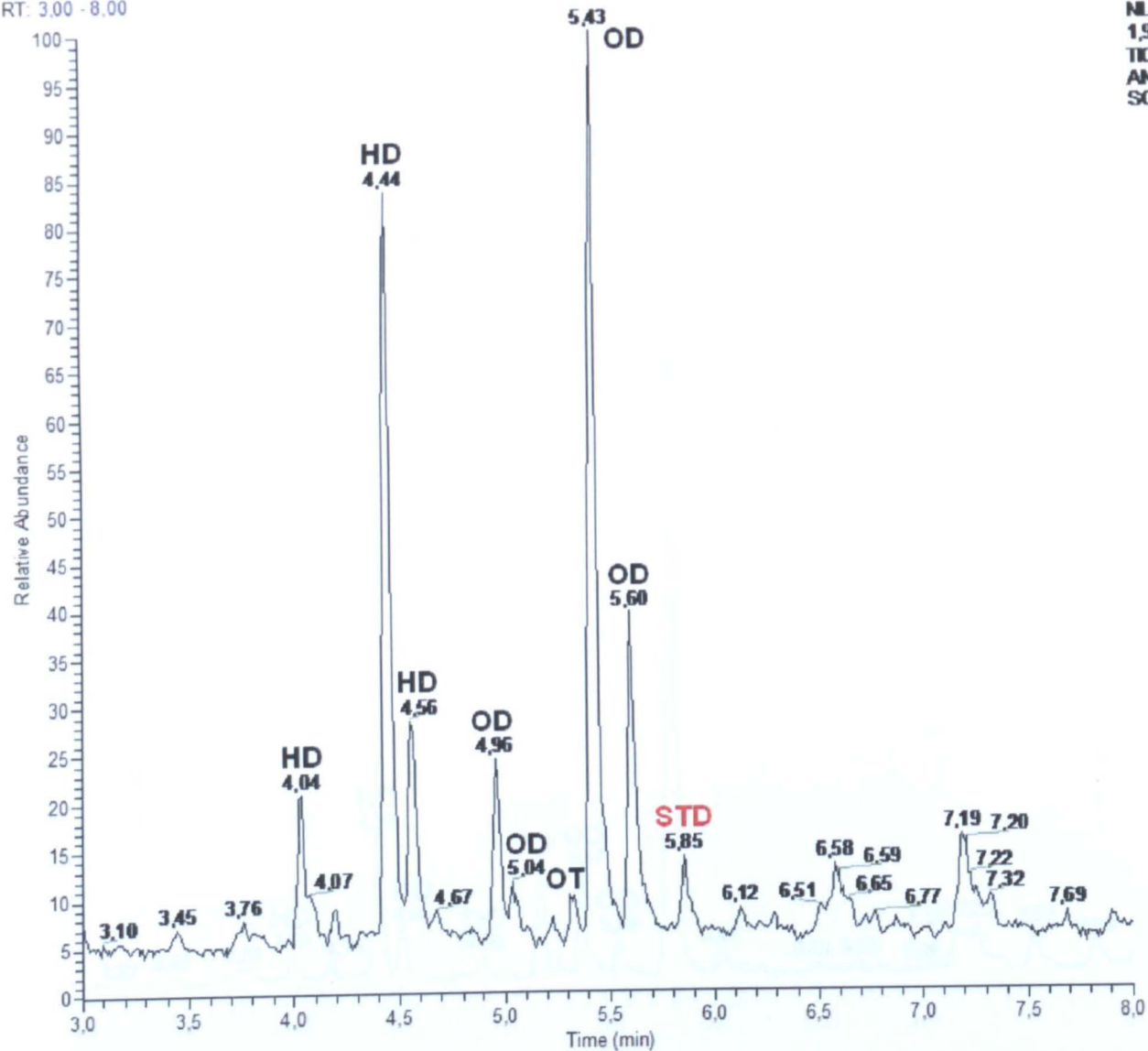
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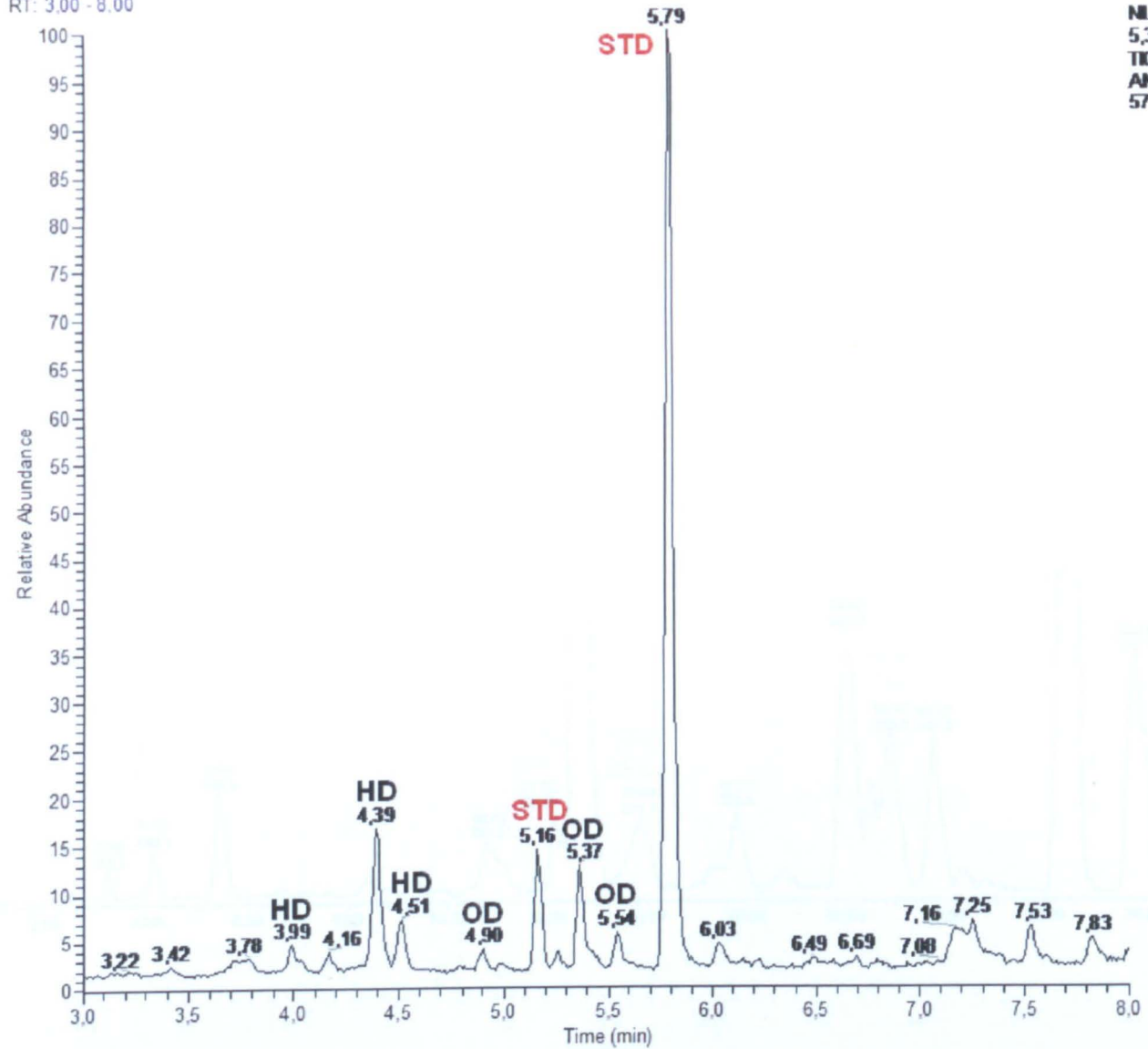


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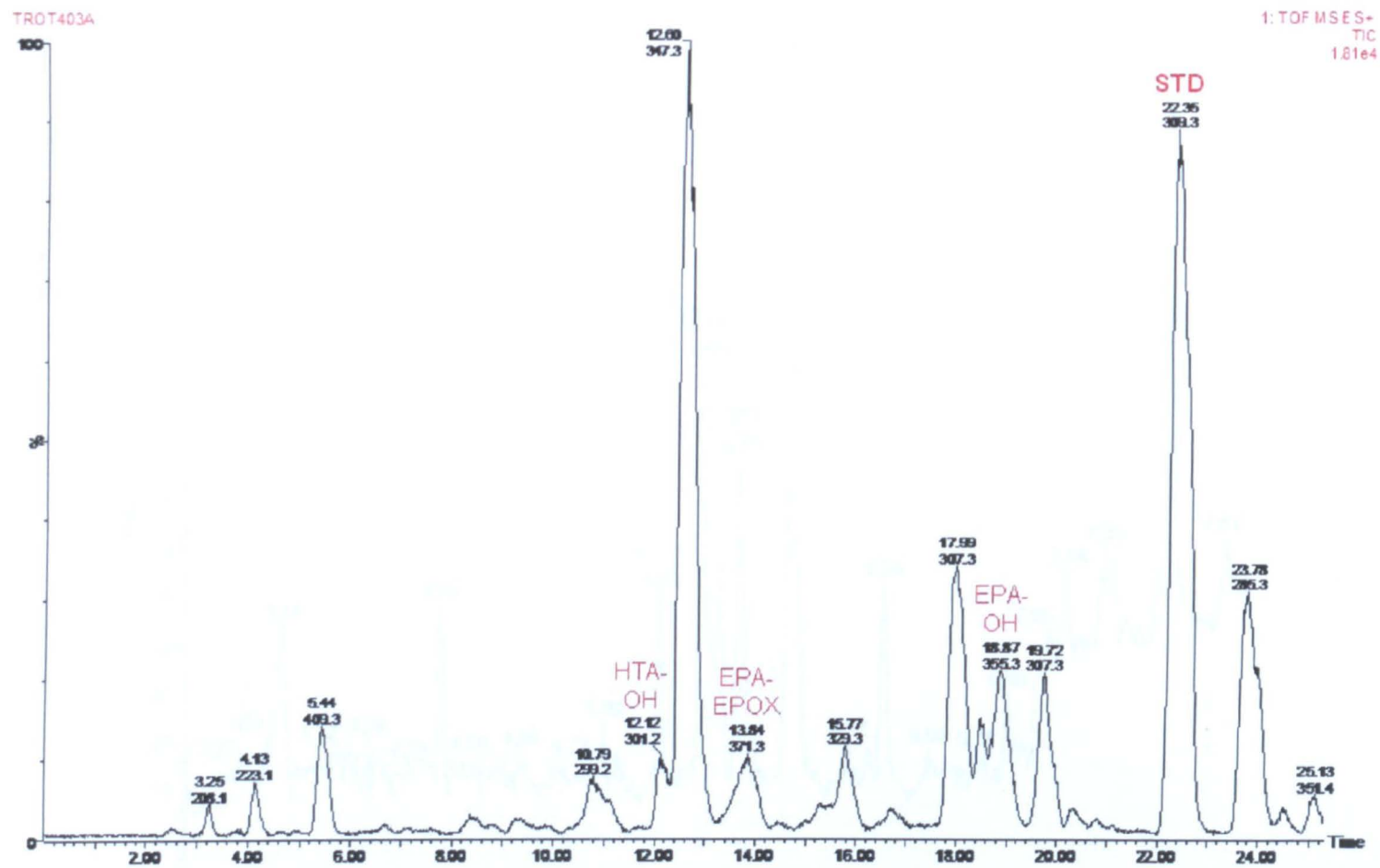


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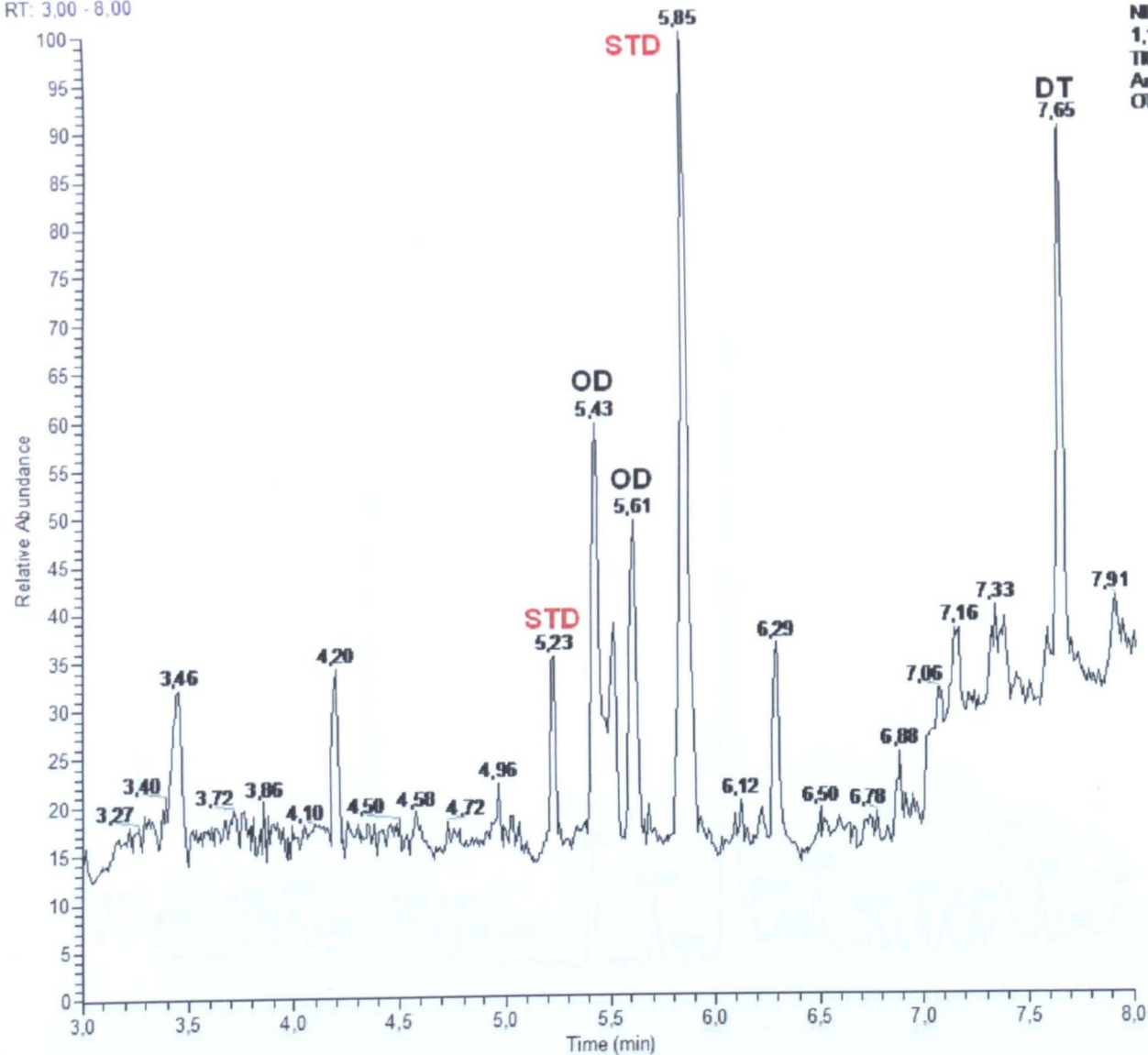
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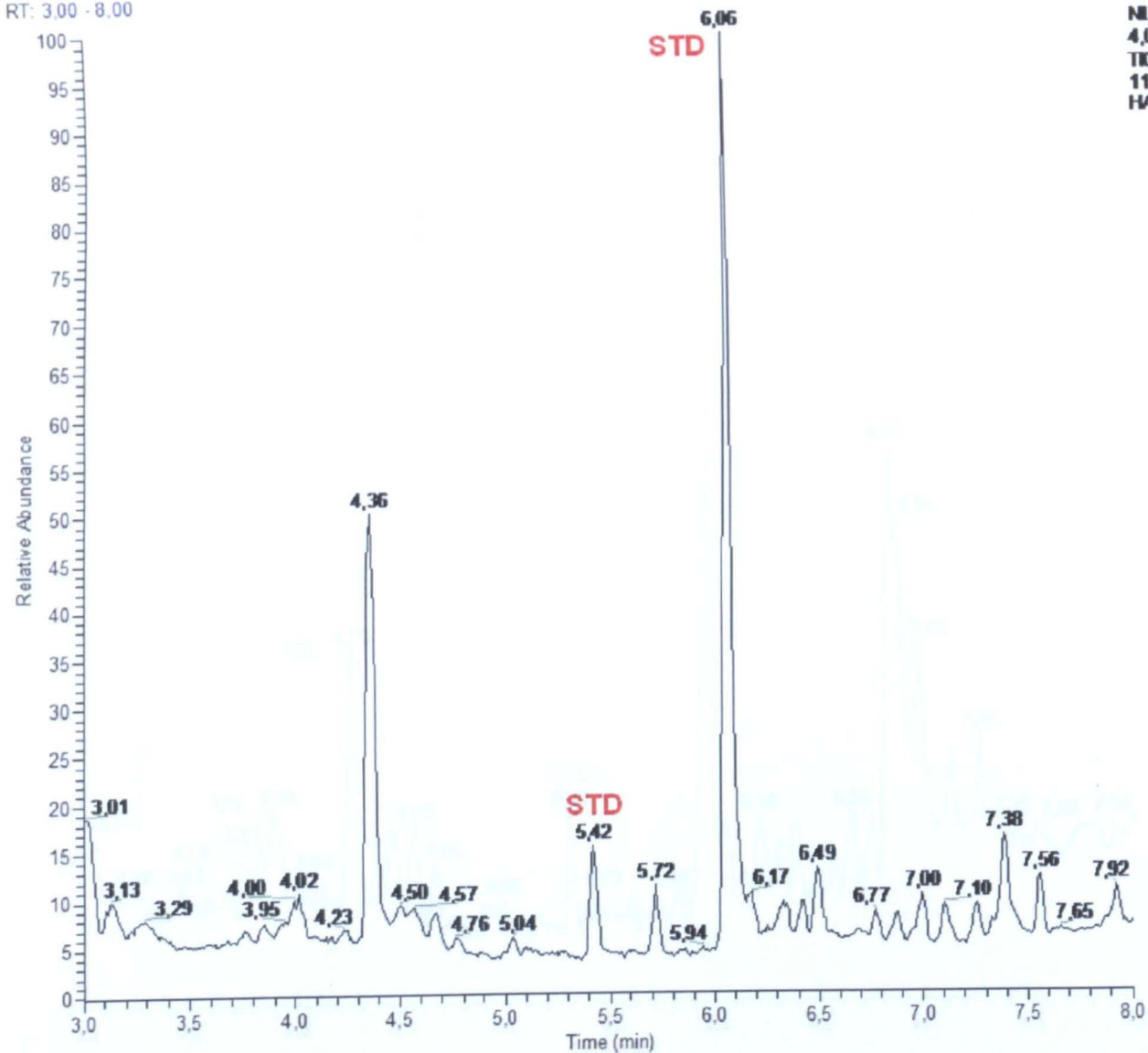


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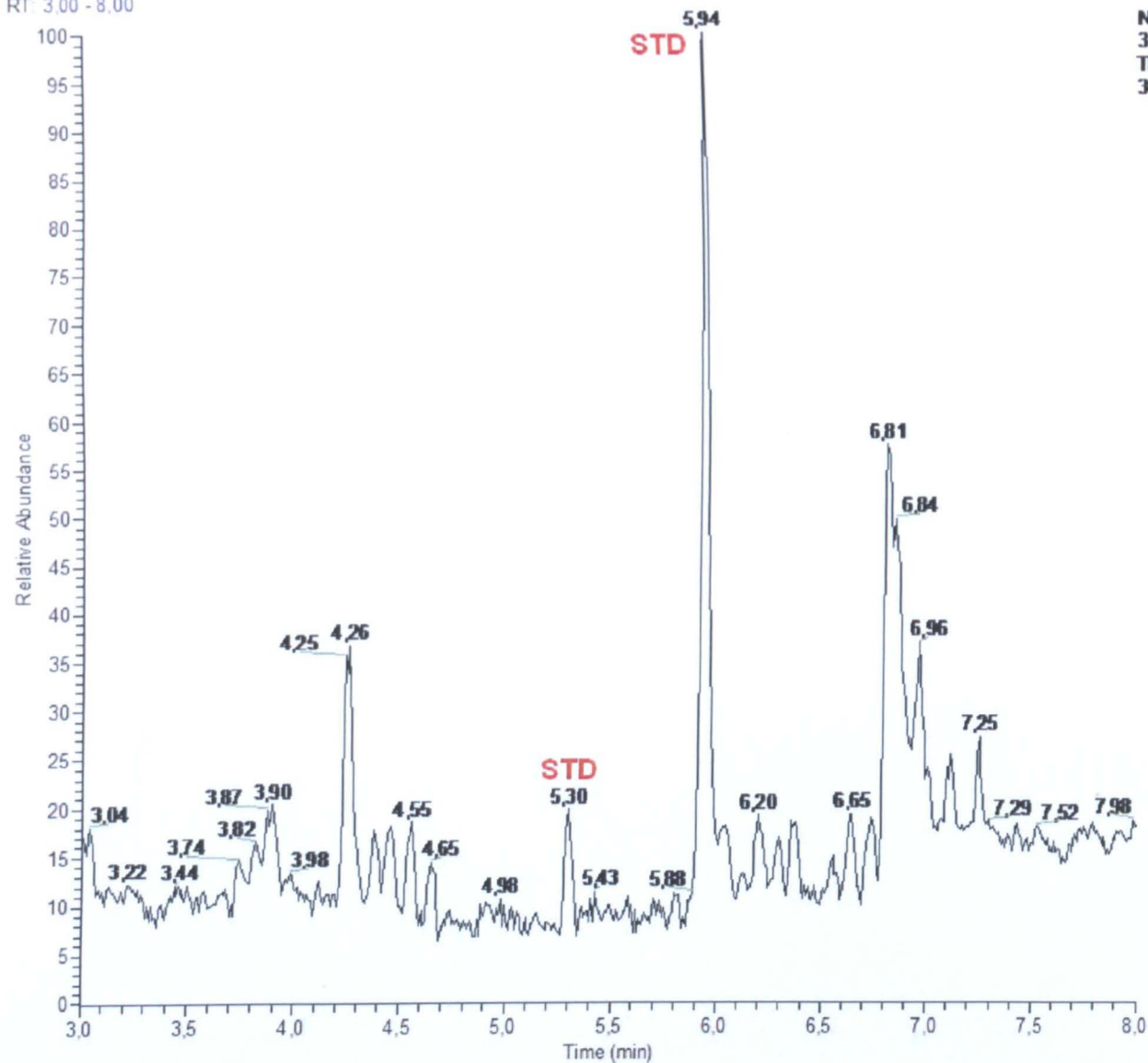
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